Bioproduction of coenzyme Q_{10} in fission yeast *Schizosaccharomyces pombe*

（分裂酵母 ***Schizosaccharomyces pombe*** でのコエンザイム Q_{10} の生産）

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Contents

Abbreviations

CDP-ME	4-diphosphocytidyl-2-*C*-methyl-D-erythritol
CDP-ME2P	4-diphosphocytidyl-2-*C*-metyl-D-erythritol 2-phosphate
CoQ	coenzyme Q
DAHP	3-deoxy-D-arabino-heptulosonate 7-phosphate
DMAPP	dimethylallyl diphosphate
DPP	decaprenyl diphosphate
DXP	D-1-deoxyxylulose-5-phosphate
E4P	erythrose-4-phosphate
FPP	farnesyl diphosphate
GAP	glyceraldehyde 3-phosphate
GGPP	geranylgeranyl diphosphate
HMBPP	1-hydroxyl-2-methyl-2-(*E*)-butenyl-4-diphosphate
HMG-CoA	3-hydroxy-3-methylflutaryl-CoA
IPP	isopentenyl diphosphate
ME-CDP	2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate
MEP	2-*C*-methyl-D-erythritol 4-phosphate
MVA	mevalonate
pABA	*p*-aminobenzoic acid
PEP	phosphoenolpyruvate
PHB	*p*-hydroxybenzoate
PM	pombe minimum
UMP	uridine monophosphate

Chapter 1

General introduction

Coenzyme Q biosynthesis

Coenzyme Q (CoQ), also called ubiquinone, is a well known component of the electron transfer system in eukaryotic cells and most prokaryotes. CoQ consists of an isoprenoid side chain and a quinone ring, and delivers electrons through the conversion of ubiquinol (reduced form) to ubiquinone (oxidized form) (Fig. 1-1). CoQ also plays additional roles distinct from the electron transfer system. For example, CoQ acts as a fat-soluble antioxidant which contributes to the removal of lipid peroxidation, and plays the role of the electron donor during disulfide bond formation in *Escherichia coli* (Inaba 2009), and its reduction is coupled to sulfide oxidation in *Schizosaccharomyces pombe* and human (Zhang et al. 2008b; Ziosi et al. 2016). Moreover, CoQ is required for *de novo* synthesis of UMP in many eukaryotes (López-Martín et al. 2007; Matsuo et al. 2013).

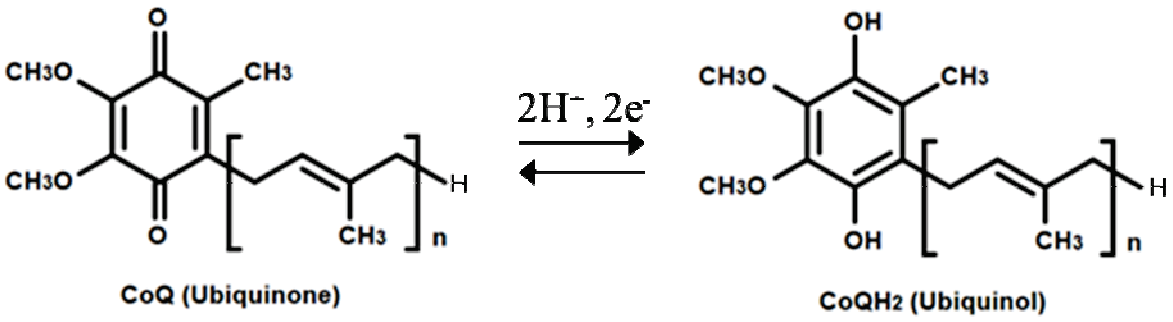

Fig. 1-1 Structure of ubiquinone and ubiquinol
Coenzyme Q (CoQ) consists of a hydrophobic isoprenoid side chain and a quinone ring, and delivers electrons through the conversion of ubiquinol (reduced form) and ubiquinone (oxidized form)

Living organisms possess different species of CoQ, and these are classified according to the length of the isoprenoid side chain. For example, human and *S. pombe* produce CoQ with ten isoprene units (CoQ_{10}), *E. coli* produces CoQ_8, and *Saccharomyces cerevisiae* produces CoQ_6. Side chain length is defined by polyprenyl diphosphate synthases (Okada et al. 1996; Okada et al. 1998a). Among various types of CoQ, human type CoQ_{10} is commercially popular as a food supplement. Therefore understanding of biosynthesis of CoQ is important for its application to produce CoQ_{10}.

The biosynthetic pathway of CoQ has been elucidated primarily through genetic analysis of *E. coli* and *S. cerevisiae* mutants (Fig. 1-2). *E. coli ubi* (*ubiA-J* and *ubiX*) mutants and *S. cerevisiae COQ* (*COQ1-COQ9*) mutants, which are unable to synthesize CoQ, were used to define the biosynthetic pathway of CoQ (Kawamukai 2002; Meganathan 2001; Tran et al. 2007; Tzagoloff et al. 1990; Hajj Chehade et al. 2013; Kawamukai 2016). Starting from the condensation of the isoprenoid side chain to *p*-hydroxybenzoate (PHB), the benzoquinol ring is then modified by methylations, decarboxylation and hydroxylations (Kawamukai 2002). At least eleven genes (*ubiA-J*, *ubiX*) in *E. coli*, nine genes (*COQ1-COQ9*) in *S. cerevisiae* and ten genes (*dps1*, *dlp1*, *ppt1*, *coq3-coq9*) in *S. pombe* are required for CoQ biosynthesis (Hajj Chehade et al. 2013; Kawamukai 2009; Suzuki et al. 1997; Uchida et al. 2000; Saiki et al. 2003a; Saiki et al. 2003b); however, the functions of the *COQ4* and *COQ9* genes have not yet been resolved. Table 1-1 shows a comparison of the CoQ biosynthetic genes from three species, namely *S. cerevisiae*, *S. pombe* and *E. coli*. The order of the first three steps of the CoQ biosynthetic pathway differs in *E. coli* and *S. cerevisiae* (Fig. 1-2). It has been suggested that Coq polypeptides form a complex in the mitochondria in *S. cerevisiae* (He et al. 2014), and complexes that lack any of the components are unstable (Marbois et al. 2005; Gin et al. 2005). This is one reason why the functions of some of the components are as yet unknown.

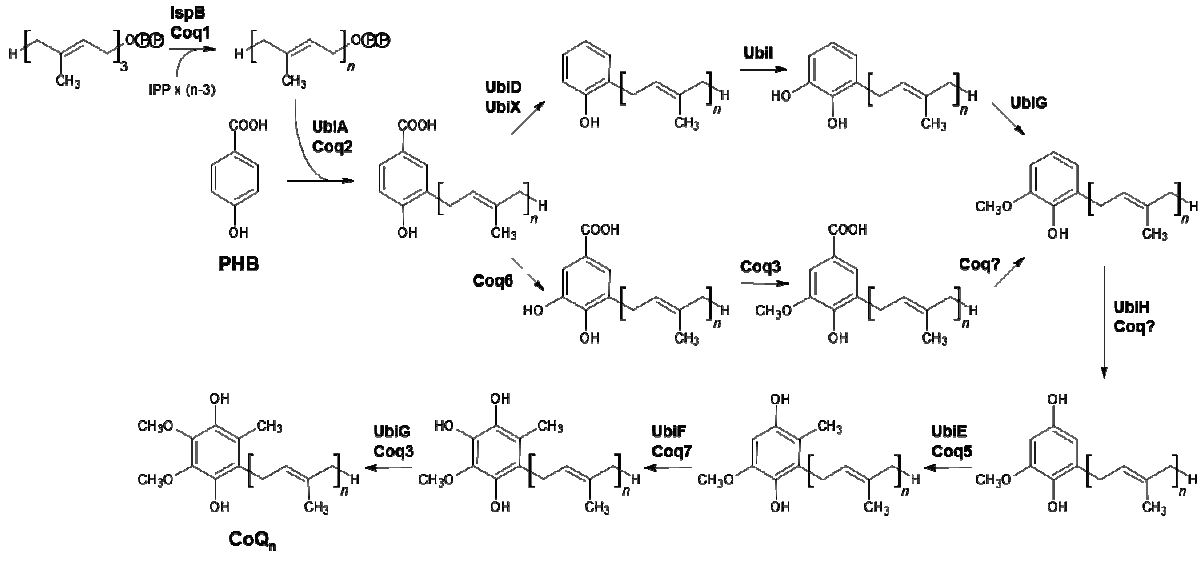

Fig. 1-2 The pathway of CoQ biosynthesis
The pathways of CoQ biosynthesis in *E. coli* and *S. cerevisiae* are shown. All *E. coli ubi* genes involved in the CoQ synthesis are assigned in the pathway, whereas two steps in the pathway and three *COQ* genes, namely *COQ4*, *COQ8* and *COQ9*, are not functionally defined in *S. cerevisiae*.

Table 1-1 Comparison of CoQ biosynthetic genes among yeasts and *E. coli*

Function	*S. cerevisiae*	*S. pombe*	*E. coli*
Polyprenyl diphosphate synthase	*COQ1*	*dps1* + *dlp1*	*ispB*
PHB-polyprenyl diphosphate transferase	*COQ2*	*ppt1*	*ubiA*
O-methyltransferase	*COQ3*	*coq3*	*ubiG*
Unknown	*COQ4*	*coq4*	-
C-methyltransferase	*COQ5*	*coq5*	*ubiE*
Monooxygenase	*COQ6*	*coq6*	*ubiI*
Monooxygenase	*COQ7*	*coq7*	*ubiF*
Protein kinase	*COQ8* (*ABC1*)	*coq8*	(*ubiB*)
Unknown	*COQ9*	*coq9*	-

p-Hydroxybenzoate biosynthesis

In *E. coli*, the aromatic ring of the CoQ molecule is derived from the shikimate pathway, which starts with the condensation of erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP) (Fig. 1-3). The shikimate pathway leads to the production of the aromatic intermediate chorismate, a precursor for several essential aromatic molecules (Herrman et al. 1999). Chorismate is converted to *p*-hydroxybenzoate (PHB) by the chorismate pyruvate-lyase UbiC. The *ubiC* gene of *E. coli* is located immediately upstream of *ubiA* encoding PHB-octaprenyltransferase on the chromosome and is controlled by one operon; collectively called *ubiCA*.

There are two pathways for the biosynthesis of PHB in yeast. One is the biosynthesis of PHB from chorismate like *E. coli* and the other is from tyrosine as found in other higher eukaryotes (Fig. 1-4) (Meganathan 2001). Yeast usually synthesizes PHB from chorismate. Mutants impaired in the shikimate pathway are not able to biosynthesize shikimate or chorismate, and as a result PHB is not biosynthesized. However, since tyrosine in the medium can be used, CoQ is synthesized. On the other hand, animal cells are able to biosynthesize PHB only from tyrosine not from chorismate, because they have no shikimate pathway. Tyrosine is synthesized from phenylalanine which is an essential amino acid.

Some recent studies in *S. cerevisiae* have shown that pABA (*p*-aminobenzoic acid), which is also synthesized from chorismate, is used as a raw material for the quinone ring of CoQ (Marbois et al. 2010), and fatty aldehyde dehydrogenase *hfd1* gene in the biosynthesis of PHB from tyrosine is essential for CoQ biosynthesis (Payet et al. 2016).

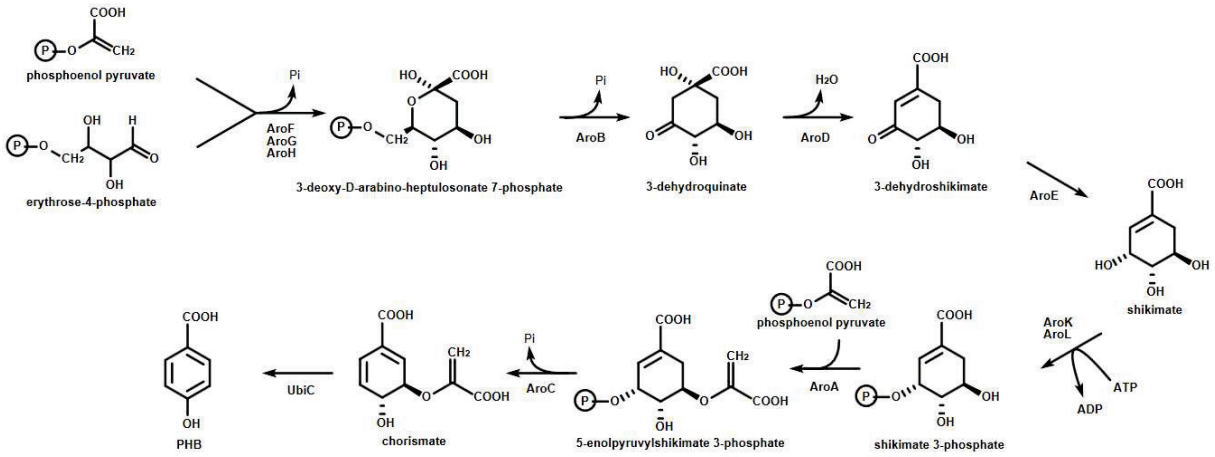

Fig. 1-3 Formation of *p*-hydroxybenzoate (PHB) via shikimate pathway

In *E. coli*, shikimate pathway starts with the condensation of erythrose-4-phosphate and phosphoenolpyruvate, and leads to the production of chorismate. Chorismate is converted to PHB by the chorismate pyruvate-lyase UbiC.

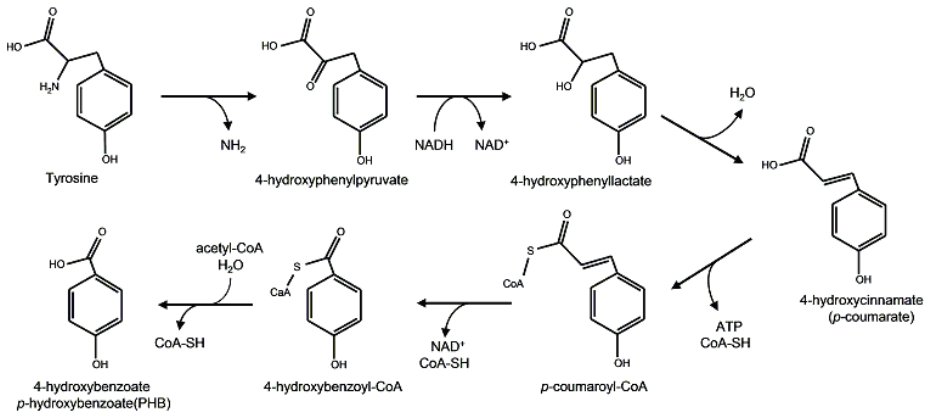

Fig. 1-4 Formation of p-hydroxybenzoate (PHB) from tyrosine

Tyrosine is converted into *p*-coumarate by the ammonia-lyase and *p*-coumarate is activated by ligation with CoA for its subsequent shortening into PHB

Isoprenoid biosynthesis

Isoprenoid compounds numerously exist in nature possess various functions in organisms. Some of the important compounds of isoprenoids include sterols, dolichols, carotenoids, prenylated proteins, terpenoids and CoQ, menaquinone and plastoquinone (Grünler et al. 1994). The isoprenoid compounds are synthesized by condensation of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) by polyprenyl diphosphate synthases (poly-PDS). IPP is a basic compound for isoprenoids synthetic pathway and is synthesized through the mevalonate pathway (MVA pathway) in eukaryotes and the non-mevalonate pathway (MEP pathway) in prokaryotes or plant plastids.

In MVA pathway, IPP synthesis starts from by condensation of acetyl-CoA with acetoacetyl-CoA and subsequently 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), mevalonate, and mevalonate- 5-phosphate and mevalonate-5-diphosphate are synthesized (Fig. 1-5) (Bloch 1992). Synthesized IPP is isomerized to DMAPP by IPP isomerase. In MEP pathway, IPP is derived from glyceraldehyde 3-phosphate (GAP) and pyruvate via D-1-deoxyxylulose-5-phosphate (DXP), 2-*C*-methyl-D-erythritol 4-phosphate (MEP), 4-diphosphocytidyl-2-*C*-methyl-D-erythritol (CDP-ME), 4-diphosphocytidyl-2-*C*-metyl-D-erythritol 2-phosphate (CDP-ME2P), 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (ME-CDP) and 1-hydroxyl-2-methyl-2-(*E*)-butenyl-4-diphosphate (HMBPP). In the last step of MEP pathway, HMBPP is converted to both IPP and DMAPP (Fig.1-6) (Eisenreich et al. 2004; Choi et al. 2005; Jeya et al. 2010; Lu et al. 2013).

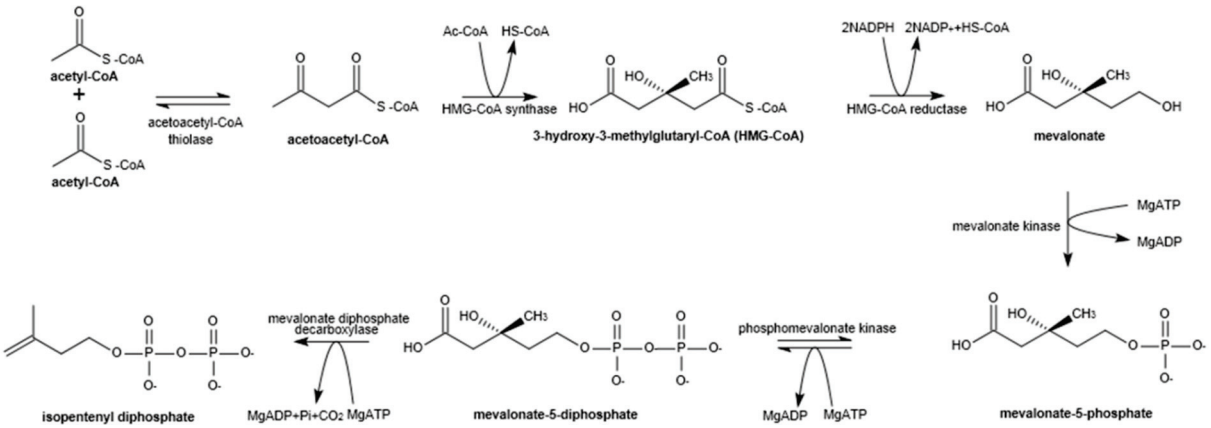

Fig. 1-5 Formation of isopentenyl diphosphate (IPP) via MVA pathway
In the MVA pathway, IPP is synthesized by condensation of three acetyl-CoA via acetoacetyl-CoA, 3-hydroxy-3-methylglutaryl-CoA, mevalonate, mevalonate-5-phosphoate and mevalonate-5-diphosphoate. The MVA pathway exists in plant cytosol and eukaryotes.

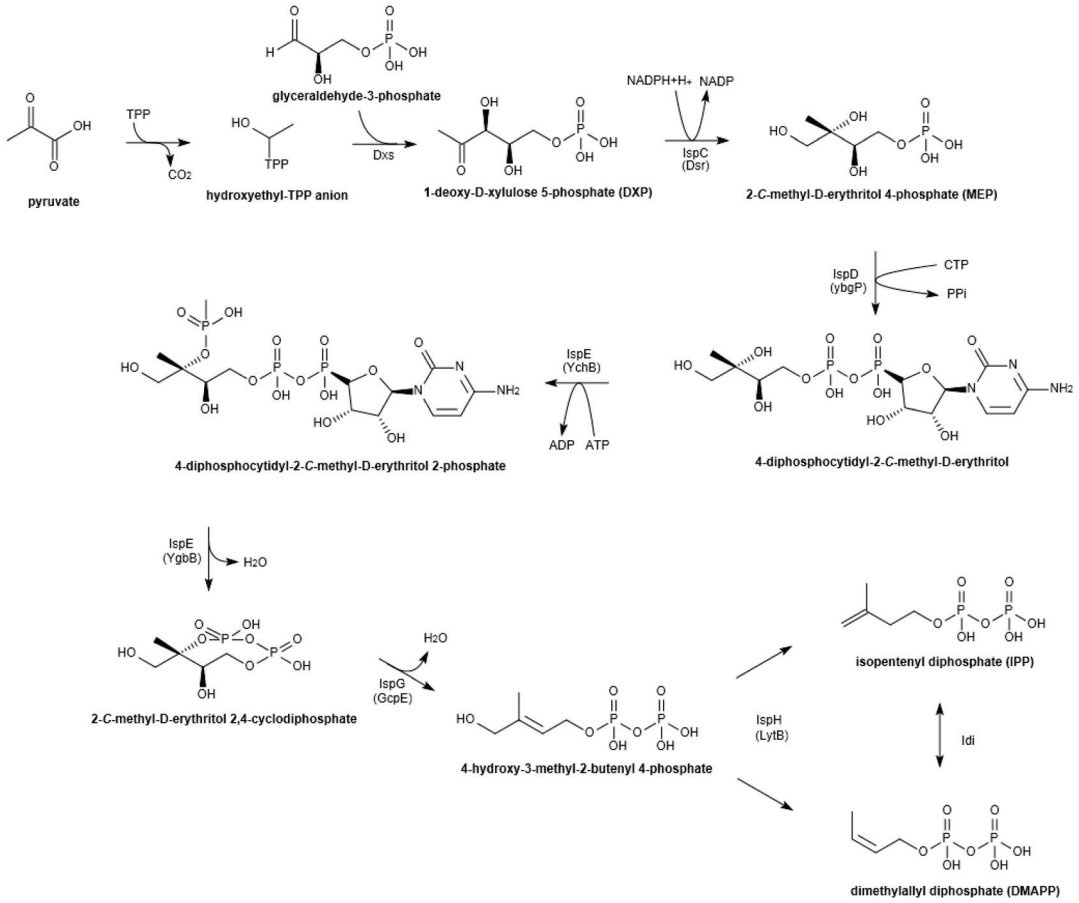

Fig. 1-6 Formation of isopentenyl diphosphate (IPP) via MEP pathway
In the MEP pathway, IPP is synthesized by condensation of glyceraldehyde 3-phosphate (GAP) and pyruvate via D-1-deoxyxylulose-5-phosphate (DXP), 2-*C*-methyl-D-erythritol 4-phophate (MEP), 4-diphosphocytidyl-2-*C*-methyl-D-erythritol (CDP-ME), 4-diphosphocytidyl-2-*C*-metyl-D-erythritol 2-phosphate (CDP-ME2P), 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (ME-CDP) and 1-hydroxyl-2-methyl-2-(*E*)-butenyl-4-diphosphate (HMBPP). The MEP pathway exists in plant plastids and bacteria.

Condensation of IPP and DMAPP is catalyzed by poly-PDS with various chain length (Fig. 1-7). Poly-PDSs can be classified into several types according to their chain length of final products. Short-chain poly-PDSs (C_{15}, C_{20}), such as farnesyl diphosphate (FPP) synthase and geranylgeranyl diphosphate (GGPP) synthase, have been identified in many organisms ranging from bacteria through to plants and mammals (Ye et al. 2007; Liang et al. 2002; Kainou et al. 1999; Okada et al. 2000). Long-chain poly-PDSs (C_{30}–C_{50}), represented by Coq1 in *S. cerevisiae*, catalyzes the condensation of FPP or GGPP, which acts as a primer, with IPP. Analyses of the long-chain poly-PDSs have been relatively limited to those in several bacteria, yeasts, trypanosomes, plants and mammals (Saiki et al. 2003a; Saiki et al. 2005; Kainou et al. 2001; Jun et al. 2004; Asai et al. 1994; Ferella et al. 2006; Okada et al.

1997a). These enzymes possess seven conserved regions, including two DDXXD motifs that are binding sites for the substrates in association with Mg^{2+} (Koyama 1999). Various mutants of poly-PDSs were constructed, and the most significant site for the determination of the length was the fifth amino acid position from the first aspartate-rich region, as proposed from the analysis of FPP synthase and GGPP synthase (Okada et al. 1998b). It was also found that the formation of an IspB dimer is essential for the determination of the length (Kainou et al. 2001). Analyses of Coq1 (hexaprenyl diphosphate synthase) from *S. cerevisiae* and the solanesyl diphosphate synthase from *Arabidopsis thaliana* suggest that the long-chain trans-poly-PDS that synthesize the CoQ side chain tend to be homomeric enzymes (Zhang et al. 2008a; Jun et al. 2004; Hirooka et al. 2003). However, the decaprenyl diphosphate synthase from *S. pombe* is a heterotetramer of Dps1 (decaprenyl diphosphate synthase) and Dlp1 [D(aspartate)-less polyprenyl diphosphate synthase] (Saiki et al. 2003a). Dlp1 lacks the aspartate-rich motifs and is bound to Dps1 to constitute the heteromeric enzyme (Saiki et al. 2003a; Saiki et al. 2005). This heterotetramer composition of poly-PDSs was also found in human and mouse (Saiki et al. 2005).

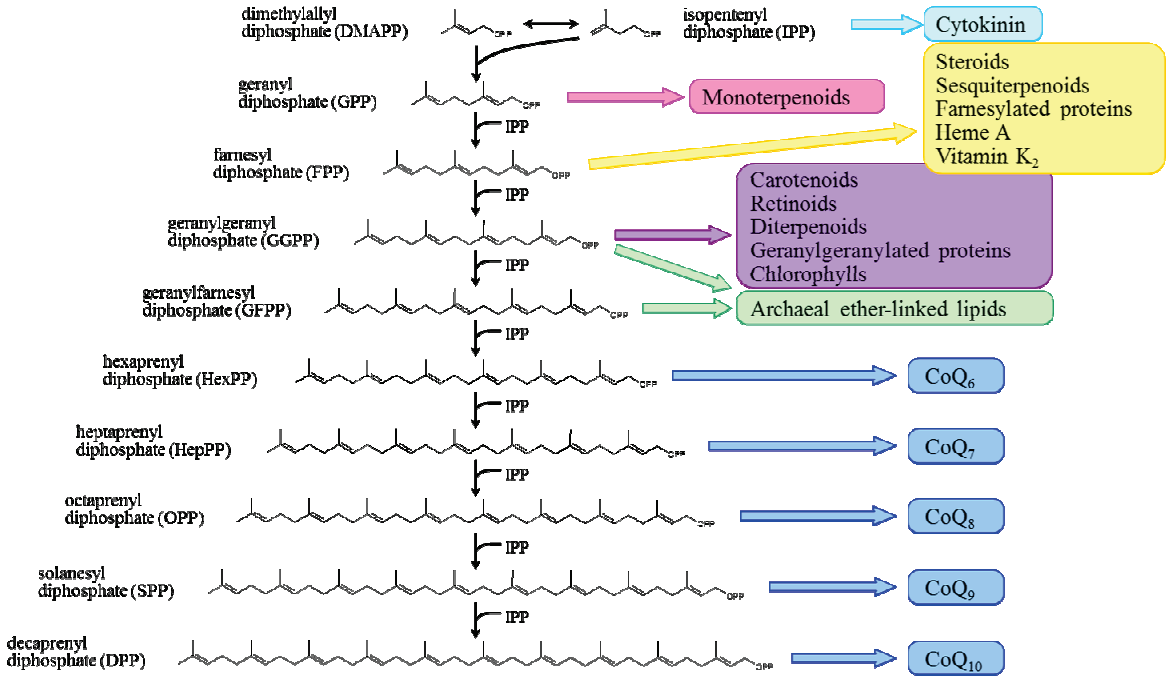

Fig. 1-7 The synthesis of various isoprenoids

Various isoprenoid chain synthesized by condensation of DMAPP and optional number of IPP. DMAPP is a C_5 unit compound that serves as the precursor to condense multiple units of IPP. Isoprenoid with more than C_{25} unit are generally used for the synthesis of the side chain of CoQ.

Bioproduction of CoQ_{10} in microorganisms

CoQ_{10} has attracted attention in recent years as a medicine or health-promoting additive to foods and cosmetics, and the demand for CoQ_{10} has risen accordingly. Several approaches have been used to improve the fermentative production of CoQ_{10} (Cluis et al. 2007), which have relied predominantly on bacterial and yeast mutants selected for their high CoQ_{10} content (Yoshida et al. 1998). Moreover, genetic engineering approaches to increase CoQ_{10} production were reported in *E. coli*, *Agrobacterium* and *Rhodobacter*. *E. coli* naturally produces CoQ_8, but expressing decaprenyl diphosphate synthase in cells that lack the endogenous *ispB* gene enables them to produce CoQ_{10} (Okada et al. 1998b). A previous study generated an engineered *E. coli* strain that expressed the *Gluconobacter suboxydans* decaprenyl diphosphate synthase gene (*ddsA*); this strain was capable of producing CoQ_{10} at concentrations up to 0.29 mg/g-dry cell weight (DCW) (Park et al. 2005). By optimizing the culture conditions, *Agrobacterium tumefaciens* capable of producing CoQ_{10} at concentrations up to 8.54 mg/g-DCW (Ha et al. 2007), and *Rhodobacter sphaeroides* can produce CoQ_{10} at concentrations up to 7.16 mg/g-DCW (Lu et al. 2014), but use of these technologies was limited in yeasts.

Composition of this thesis

The purpose of this study is to improve the productivity of CoQ_{10} in *S. pombe* which naturally produces CoQ_{10}, and to elucidate the various decaprenyl diphosphate synthases useful for production of CoQ_{10}. In this thesis, Chapter 1 describes a general introduction with backgrounds of this study. Chapter 2 describes an improvement of the productivity of CoQ_{10} in *S. pombe* by overproducing upstream biosynthetic precursors. Chapter 3 describes the molecular cloning of three fungi decaprenyl diphosphate synthase genes and their expression in *S. pombe*. Finally, the author concludes this thesis in Chapter 4.

Chapter 2

Production of CoQ_{10} in fission yeast by expression of genes responsible for CoQ_{10} biosynthesis

Abstract

Coenzyme Q_{10} (CoQ_{10}) is essential for energy production and has become a popular supplement in recent years. In this study, CoQ_{10} productivity was improved in the fission yeast *Schizosaccharomyces pombe*. Ten CoQ biosynthetic genes were cloned and overexpressed in *S. pombe*. Strains expressing individual CoQ biosynthetic genes did not produce higher than a 10% increase in CoQ_{10} production. In addition, simultaneous expression of all ten *coq* genes did not result in yield improvements. Genes responsible for the biosynthesis of *p*-hydroxybenzoate and decaprenyl diphosphate, both of which are CoQ biosynthesis precursors, were also overexpressed. CoQ_{10} production was increased by overexpression of *Eco_ubiC* (encoding chorismate lyase), *Eco_aroF*^{FBR} (encoding 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase), or *Sce_thmgr1* (encoding truncated HMG-CoA reductase). Furthermore, simultaneous expression of these precursor genes resulted in two fold increases in CoQ_{10} production.

Introduction

Coenzyme Q (CoQ), also referred to as ubiquinone, is a component of the electron transport chain that participates in aerobic cellular respiration within eukaryotic mitochondria and, as such, is essential for ATP-dependent energy production. CoQ consists of a hydrophobic isoprenoid side chain and a quinone ring, and delivers electrons through the conversion of quinol (reduced form) to quinone (oxidized form). CoQ acts as a fat-soluble antioxidant by this oxidation–reduction reaction, which contributes to the removal of lipid peroxidation. CoQ also plays additional roles distinct from the electron transfer system and antioxidant activities. For example, CoQ acts as an electron donor during disulfide bond formation in *E. coli* (Inaba 2009), and CoQ reduction is coupled to sulfide oxidation in *Schizosaccharomyces pombe* and other organisms (Uchida et al. 2000). CoQ is also required for *de novo* synthesis of UMP in eukaryotes (López-Martin et al. 2007; Matsuo et al. 2013).

Living organisms possess different species of CoQ, and these are classified according to the length of the isoprenoid side chain. For example, human and *S. pombe* produce CoQ with ten isoprene units (CoQ_{10}), *E. coli* produces CoQ_8, and *Saccharomyces cerevisiae* produces CoQ_6. Side chain length is defined by polyprenyl diphosphate synthases (Okada et al. 1996; Okada et al. 1998a). The biosynthetic pathway for the complete conversion of *p*-hydroxybenzoate (PHB) to CoQ consists of at least nine steps and eight enzymes in yeast (Fig. 2-1)

(Kawamukai 2002; Kawamukai 2009). These steps include the condensation of IPP (isopentenyl diphosphate) and transfer of the isoprenoid side chain to PHB followed by hydroxylation, methylation, and decarboxylation steps. In eukaryotes, this pathway has been most comprehensively studied to date in *S. cerevisiae* and *S. pombe*. At least nine genes (*COQ1-COQ9*) in *S. cerevisiae* and ten genes (*dps1*, *dlp1*, *ppt1*, *coq3-coq9*) in *S. pombe* are required for CoQ biosynthesis (Fig. 2-1) (Kawamukai 2016); however, the functions of the *COQ4* and *COQ9* genes have not yet been resolved.

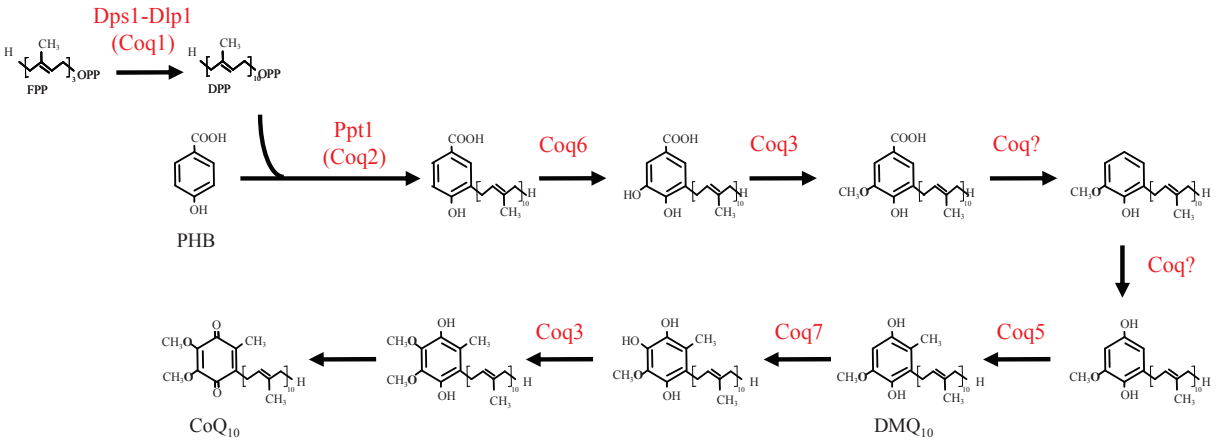

Fig. 2-1 Biosynthetic pathway of coenzyme Q (CoQ) in *S. pombe*
The pathway of CoQ biosynthesis in *S. pombe* is shown. *S. cerevisiae* protein names are shown in parantheses. At least ten genes (*dps1, dlp1, ppt1, and coq3–9*) are involved in coenzyme Q_{10} (CoQ_{10}) biosynthesis in *S. pombe*. Dps1 and Dlp1 form a heterotetramer and condense FPP (farnesyl diphosphate) with IPP (isopentenyl diphosphate) into DPP (decaprenyl diphosphate). Ppt1 is a *p*-hydroxybenzoate (PHB)-decaprenyl diphosphate transferase that catalyzes the condensation of PHB and DPP. Other enzymes catalyze modification of the quinone structure. DMQ_{10}, demethoxyubiquinone10.

CoQ_{10} has attracted attention in recent years as a medicine or health-promoting additive to foods and cosmetics, and the demand for CoQ_{10} has risen accordingly. Several approaches have been used to improve the fermentative production of CoQ_{10} (Cluis et al. 2007), which have relied predominantly on bacterial and yeast mutants selected for their high CoQ_{10} content (Yoshida et al. 1998). Moreover, genetic engineering approaches to increase CoQ_{10} production were reported in *E. coli* (22.5 mg/L, 0.29 mg/g-dry cell weight (DCW)) (Park et al. 2005; Zhu et al. 1995), *Agrobacterium tumefaciens* (548.2 mg/L, 6.92 mg/g-DCW) (Yoo et al. 2008), and *Rhodobacter sphaeroides* (93.3 mg/L, 7.16 mg/g-DCW) (Lu et al. 2014), but use of these technologies was limited in yeasts. In this study, I aimed to improve the productivity of CoQ_{10} in *S. pombe* by expression of a panel of biosynthetic genes. Since *S. pombe* naturally

produces CoQ_{10}, it is easier to manipulate gene expression to produce CoQ_{10} than other popular organisms such as *S. cerevisiae* and *E. coli*, which only produce CoQ_6 and CoQ_8, respectively. Availability of gene manipulation tools in *S. pombe* is also beneficial for using this yeast for CoQ_{10} production.

Materials and methods

Strains, media, and genetic manipulation.

The *S. pombe* strain CHP429 (*h*$^-$, *ade6-M216*, *leu1–32*, *ura4-D18*, *his7–366*) (Apolinario et al. 1993) was used in this study. Standard yeast culture media and genetic manipulations were performed as described previously (Moreno et al. 1991). *S. pombe* strains were grown in complete YES medium (0.5% yeast extract, 3% glucose, 225 mg/L adenine, 225 mg/L leucine, 225 mg/L uracil, 225 mg/L histidine, and 225 mg/L lysine hydrochloride) or PM medium (0.3% potassium hydrogen phthalate, 0.56% sodium phosphate, 0.5% ammonium chloride, 2% glucose, vitamins, minerals, and salts) (Alfa et al. 1993). The appropriate auxotrophic or antibiotic supplements were added as necessary (75 mg/L adenine, 75 mg/L leucine, 75 mg/L uracil, 75 mg/L histidine, 75 mg/L lysine, and/or 0.025 μg/m L Aureobasidin A). *E. coli* DH5α was used as a host strain for all plasmid manipulations and was grown in LB medium (1% bactotryptone, 0.5% yeast extract, and 1% NaCl; pH 7.0). Standard molecular biology protocols were followed (Sambrook et al. 1989). Restriction enzymes were used according to the suppliers' recommendations (TOYOBO Co. Ltd, Takara Bio Inc., and NEB Ltd.).

Plasmid construction.

The plasmids constructed in this study are shown in Fig. 2-2. The primers used for plasmid construction are listed in Table 2-1. pREP1-dps1-dlp1 was constructed by inserting the *dlp1* expression cassette (*nmt1* promoter—*dlp1* gene—*nmt1* terminator), which had been amplified using the nmt1-pro/PstI and nmt1-term/PstI primers from pREP1-dlp1 (Hayashi et al. 2014), into the *Pst*I site of pREP1-dps1 (Hayashi et al. 2014). pREP1-HIS7-ppt1-coq5 was constructed by inserting the *coq5* expression cassette (*nmt1* promoter—*coq5* gene—*nmt1* terminator), which had been amplified using the nmt1-pro/PstI and nmt1-term/PstI primers from pREP1-coq5 (Hayashi et al. 2014), into the *Pst*I site of pREP1-HIS7-ppt1. pREP1-HIS7-ppt1 was constructed by inserting the *ppt1* gene into the *Sal*I and *Sma*I sites of pREP1-HIS7, which was constructed by removing *LEU2* by *Hin*dIII digestion of pREP1 and

inserting *his7* into the *Sac*I site. pREP2-coq3-coq6 was constructed by inserting the *coq6* expression cassette (*nmt1* promoter—*coq6* gene—*nmt1* terminator), which had been amplified from pREP1-coq6 using the nmt1-pro/PstI and nmt1-term/PstI primers (Hayashi et al. 2014), into the *Pst*I site of pREP2-coq3. pREP2-coq3 was constructed by inserting the *coq3* gene into the *Sal*I and *Sma*I sites of pREP2. pREP1-AUR1-coq4-coq8 was constructed by inserting the *coq8* expression cassette (*nmt1* promoter—*coq8* gene—*nmt1* terminator), which had been amplified from pREP1-coq8 using the nmt1-pro/PstI and nmt1-term/PstI primers (Hayashi et al. 2014), into the *Pst*I site of pREP1-AUR1-coq4. pREP1-AUR1-coq4 was constructed by inserting the *coq4* gene into the *Bam*HI and *Sma*I sites of pREP1-AUR1, which was constructed by removing.

LEU2 by *Hin*dIII digestion of pREP1 and inserting *aur1* (Hashida-Okado et al. 1998) into the *Sac*I site. pREP1-KAN-coq7-coq9 was constructed by inserting the *coq9* expression cassette (*nmt1* promoter—*coq9* gene—*nmt1* terminator), which had been amplified from pREP1-coq9 using the nmt1pro-n-SphI and nmt1term-c-SphI (Hayashi et al. 2014), into the *Sph*I site of pREP1-KAN-coq7. pREP1-KAN-coq7 was constructed by inserting the *coq7* gene (Miki et al. 2008) into the *Sal*I and *Sma*I sites of pREP1-KAN, which was constructed by removing *LEU2* by *Hin*dIII digestion of pREP1 and inserting kan^r into the *Sac*I site.

Each gene located upstream of CoQ biosynthesis was amplified using primers containing restriction sites, digested with restriction endonucleases, and then cloned into the appropriate sites of the desired pREP1, pREP2, or pREP1-HIS7 vector. A single coding mutation (Pro148Leu) was introduced into *E. coli* 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase gene, by overlapping PCR, to make the Eco_aroF^{FBR} gene (Weaver et al. 1990). In the first step, two separate PCRs were used to generate the primary PCR products, designated PCR 1a and PCR 1b. Both primary PCR products contained the desired mutation as well as overlap regions that were attached to the end of the sequence. PCR 1a was performed with primers Eco_aroF-SalI_Fw and Eco_aroF_C443T-Rv, and PCR 1b was performed with primers Eco_aroF_C443T-Fw and Eco_aroF-BamHI_Rv. These primary PCR products were overlapped in the second PCR. Overlapping PCR was also used to introduce double mutations (Asn66Lys, Ile152 Met) into mevalonate kinase from *S. cerevisiae* to make the Sce_mvk^{FBR} gene (Bai et al. 2011). Primary PCR was performed with primers Sce_mvk-SalI_Fw and Sce_mvk_T198G-Rv, Sce_mvk_T198G-Fw and Sce_mvk_T456G-Rv, and Sce_mvk_T456G-Fw and Sce_mvk-BamHI_Rv. These primary PCR products were overlapped in the second round of PCR.

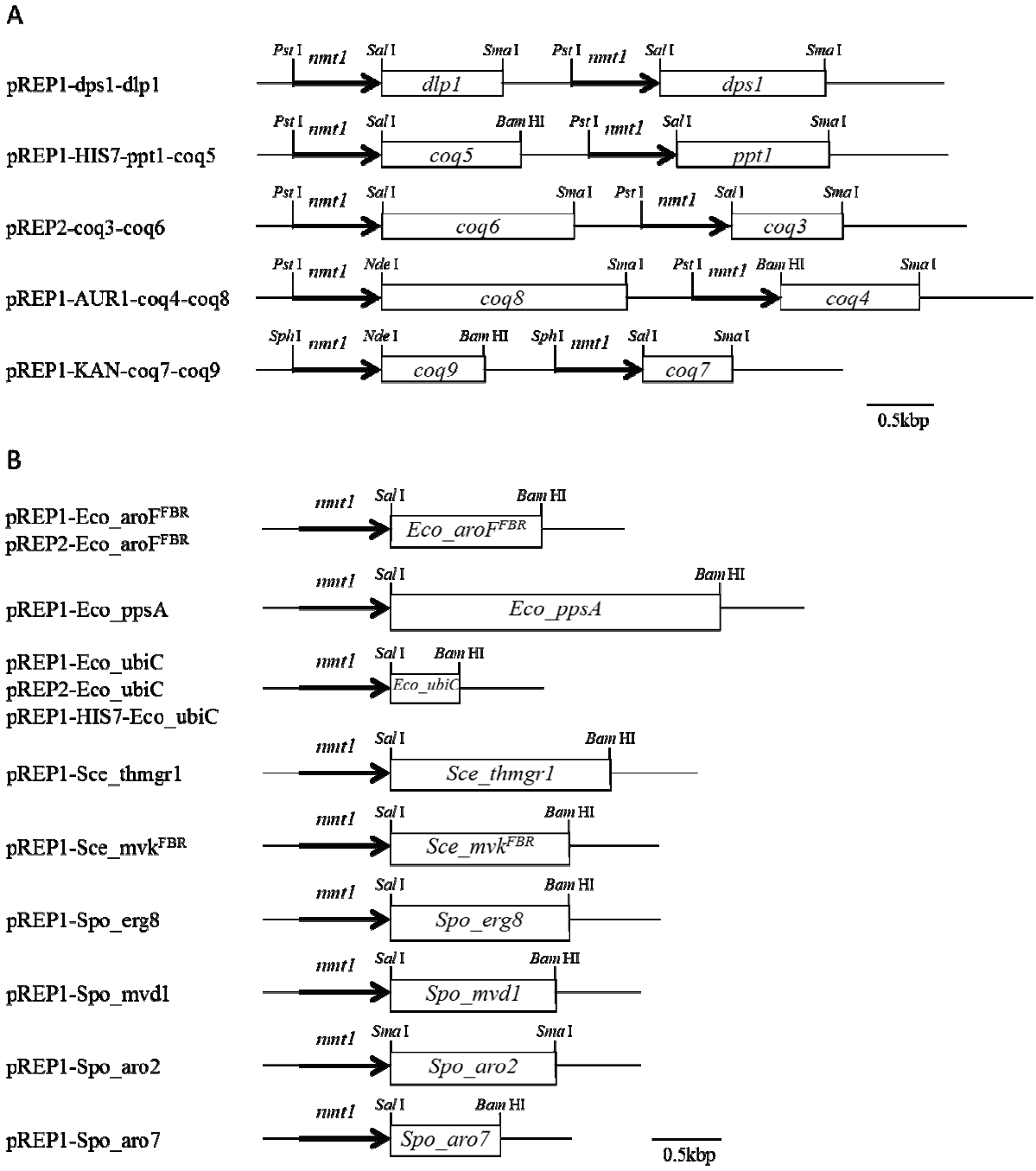

Fig. 2-2 Construction of expression plasmids

(A) To simultaneously overexpress multiple CoQ biosynthetic genes, five kinds of plasmids that expressed two genes on the same plasmid were constructed. (B) Genes encoding CoQ biosynthesis precursors were inserted into the pREP1, pREP2, or pREP1-HIS7 vector under the control of the *nmt1* promoter.

Table 2-1 Primers used for gene amplification

Name	Sequence
nmt1-pro/PstI	5′-AACTGCAGGTCGATCGACTCTAGAG-3′
nmt1-term/PstI	5′-AACTGCAGGGATTATTTCACTTC-3′
nmt1pro-n-SphI	5′-CTATGACCATGATTACGCCAAGC-3′
nmt1term-c-SphI	5′-AAAGCATGCAGGTCGACGGATC-3′
PUC119L-C	5′-ACAGCTATGACCATGATTACGCCAAG-3′
ars1L-N	5′-AAGTCACTATGTCCGAGTGGTTAAGGAG-3′
Sce_thmgr1-SalI_Fw	5′-ACGCGTCGACAATGGCTGCAGACCAATTGGT-3′
Sce_thmgr1-BamHI_Rv	5′-ACGCGGATCCTTAGGATTTAATGCAGGTGACG-3′
Sce_mvk-SalI_Fw	5′-ACGCGTCGACAATGTCATTACCGTTCTTAACTTC-3′
Sce_mvk-BamHI_Rv	5′-ACGCGGATCCTTATGAAGTCCATGGTAAATTCG-3′
Sce_mvk_T198G-Fw	5′-GTGGTCCATCAAgGATTTCAATGCC-3′
Sce_mvk_T198G-Rv	5′-GGCATTGAAATCcTTGATGGACCAC-3′
Sce_mvk_T456G-Fw	5′-AAGCGCCTCTATgTCTGTATCACTG-3′
Sce_mvk_T456G-Rv	5′-CAGTGATACAGAcATAGAGGCGCTT-3′
Eco_aroF-SalI_Fw	5′-ACGCGTCGACAATGCAAAAAGACGCGCTGAAT-3′
Eco_aroF-BamHI_Rv	5′-ACGCGGATCCTTAAGCCACGCGAGCCGTCA-3′
Eco_aroF_C443T-Fw	5′-GGAAGCGTTAGATCtGAATAGCCCGCAAT-3′
Eco_aroF_C443T-Rv	5′-ATTGCGGGCTATTCaGATCTAACGCTTCC-3′
Eco_ubiC-SalI_Fw	5′-ACGCGTCGACAATGTCACACCCCGCGTTAAC-3′
Eco_ubiC-BamHI_Rv	5′-ACGCGGATCCTTAGTACAACGGTGACGCCG-3′
Eco_ppsA-SalI_Fw	5′-ACGCGTCGACAATGTCCAACAATGGCTCGTC-3′
Eco_ppsA-BamHI_Rv	5′-ACGCGGATCCTTATTTCTTCAGTTCAGCCAGG-3′
Spo_erg8-SalI_Fw	5′-ACGCGTCGACAATGAAAGTGTAAGTTGAAACCA-3′
Spo_erg8-BamHI_Rv	5′-ACGCGGATCCTTATTCTACGGCTAGCCCAT-3′
Spo_mvd1-SalI_Fw	5′-ACGCGTCGACAATGGACAAAAAGGTTTATCAATG-3′
Spo_mvd-BamHI_Rv	5′-ACGCGGATCCTTAATGACTTCCTATAAATTTAGGG-3′
Spo_aro2-SmaI_Fw	5′-ACGCCCCGGG ATGTCTTCCTTCGGCACTTTG-3′
Spo_aro2-SmaI_Rv	5′-ACGCCCCGGGTTATTGAGCATTGGGGAGTAG-3′
Spo_aro7-SalI_Fw	5′-ACGCGTCGACAATGAGTTTGGTTAATGAGAAGC-3′
Spo_aro7-BamHI_Rv	5′-ACGCGGATCCTTAAAGTAAGCGAGCTAACAAAT-3′

Note: Nucleotides in lower case indicate point mutations.

Extraction and analysis of CoQ from S. pombe.

CoQ was extracted from *S. pombe* as described previously (Saiki et al. 2003a). Briefly, crude lipid extracts were analyzed by normal phase thin layer chromatography using authentic CoQ_{10} (as the standard) and benzene on a Kieselgel 60 F_{254} plate. Following UV visualization, the band containing CoQ_{10} was collected from the plate and extracted with chloroform/methanol (1:1 v/v). The samples were dried and resolved in ethanol. Purified CoQ was analyzed further by high-performance liquid chromatography (HPLC), with ethanol as the solvent.

Results

CoQ_{10} productivity of strains overexpressing individual CoQ biosynthetic genes

Of the various yeast species that produce CoQ_{10}, *S. pombe* was the best choice for genetic engineering because of the wealth of molecular tools available and the growing body of knowledge regarding *S. pombe* CoQ biosynthesis (Hayashi et al. 2014). First, CoQ biosynthetic genes were individually overexpressed to determine their impact on CoQ production. Individual genes (*dps1*, *dlp1*, *ppt1*, and *coq3-coq9*) were cloned into the pREP1 vector for expression under a strong promoter (*nmt1*). The production of CoQ_{10} was determined after cell growth at 30 °C for 48 h in PM minimal medium containing 2 μM thiamine. Thiamine was added because overexpression of most of the *coq* genes under the *nmt1* promoter was thought to cause growth inhibition (Fig. 2-3). The production of CoQ_{10} was evaluated per 100 ml culture volume and per 10^9 cells (Fig. 2-4). A slight increase in CoQ_{10} production was seen when *dps1-dlp1* or *coq3* were overexpressed, but this was minimal. In most cases, the overexpression of individual genes did not increase CoQ_{10} production, with production lowered in some instances.

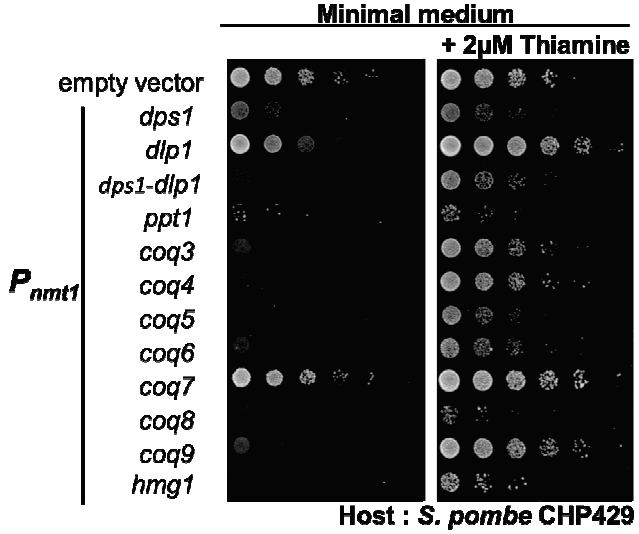

Fig. 2-3 Growth after overexpression of CoQ biosynthetic gene

Five-fold serial dilutions from 1×10^7 cells of CHP429 expressing the indicated genes were spotted onto PM minimal medium or PM minimal medium containing 2μM thiamine. Plates were incubated at 30°C for 3 days.

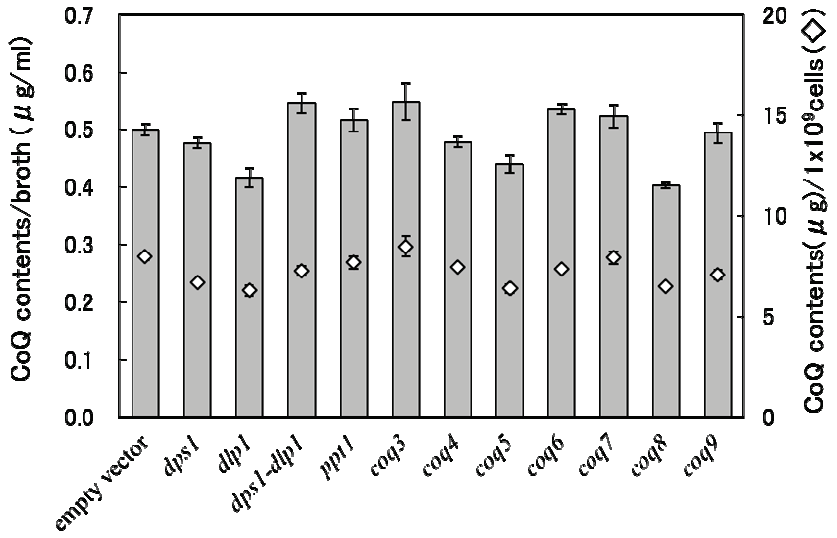

Fig. 2-4 Effect of CoQ biosynthetic gene overexpression on CoQ_{10} production
CoQ_{10} productivity in strains expressing a single CoQ biosynthetic gene or both *dps1* and *dlp1* was compared to reference strain CHP429 (empty vector). Strains were grown at 30˚C in PM minimal medium containing adenine, uracil, histidine, and thiamine. Cultures were initiated at 1×10^5 cells/ml and harvested after 48 h growth. Production of CoQ_{10} was then measured by high-performance liquid chromatography (HPLC). *Gray bars* represent CoQ_{10} content with respect to volume (μg/ml) and *open diamonds* represent CoQ_{10} contents with respect to cell count (μg/1×10^9 cells). Data are represented as the mean ± SD of three measurements.

CoQ_{10} productivity in strains co-expressing CoQ biosynthetic genes

Next, the effect of simultaneous enhancement of multiple CoQ biosynthetic genes on CoQ productivity was assessed. Plasmids were constructed that expressed two genes on the same plasmid (Fig. 2-2(A)). The *dps1* and *dlp1* genes were expressed on the plasmid pREP1-dps1-dlp1; *ppt1* and *coq5* on the plasmid pREP1-HIS7-ppt1-coq5; *coq3* and *coq6* on the plasmid pREP2-coq3-coq6; *coq4* and *coq8* on the plasmid pREP1-AUR1-coq4-coq8; and *coq7* and *coq9* on the plasmid pREP1-KAN-coq7-coq9. I initially attempted to house all these plasmids within the same yeast strain, but strain maintenance proved difficult. Strains were therefore transformed with PCR DNA fragments for chromosomal integration. Primers PUC119L-C and ars1L-N (Table 2-1) were used to amplify DNA fragments consisting of the expression cassette and selectable marker. The amplified fragments were introduced into the *S. pombe* genome by nonhomologous end joining, and transformants were screened for auxotrophic complementation and resistance to antibiotics. Production of CoQ_{10} in these

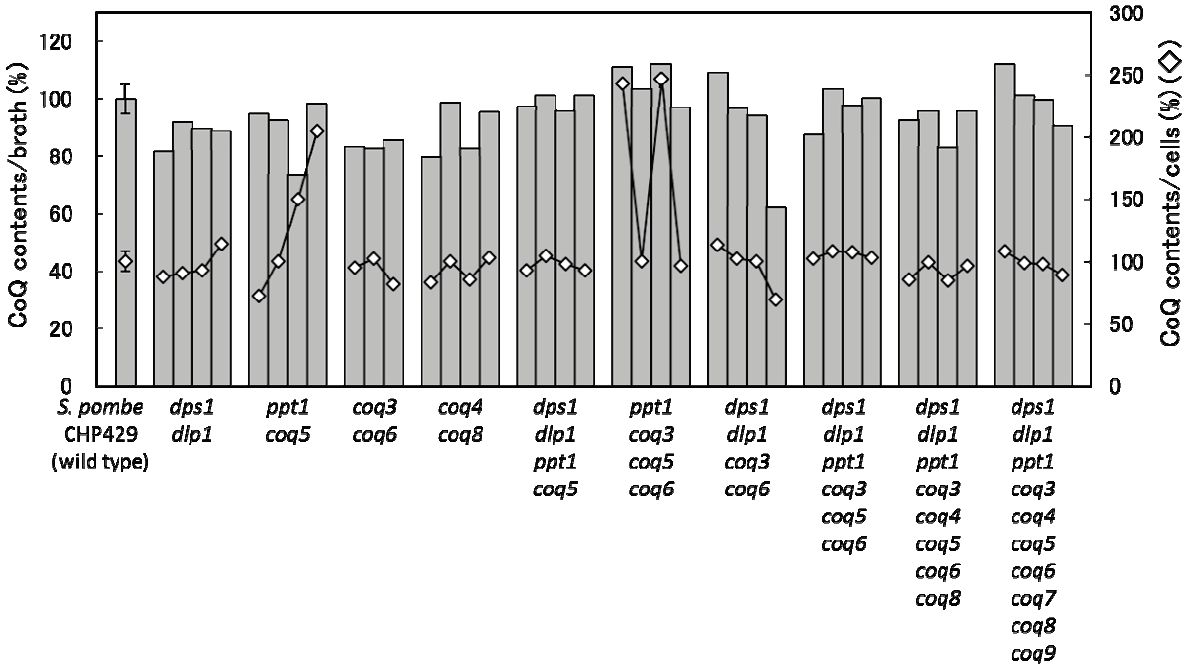

Fig. 2-5 Effect of coexpression of multiple CoQ genes on CoQ_{10} production

CoQ_{10} productivity of strains simultaneously expressing CoQ biosynthetic genes was compared to a reference strain (CHP429). Strains were grown at 30°C in YES complete medium. Cultures were initiated at 1×10^5 cells/ml and harvested after 48 h growth. Production of CoQ_{10} was then measured by HPLC. *Gray bars* represent CoQ_{10} content with respect to volume (%) and *open diamonds* represent CoQ_{10} contents with respect to cells (%). The reference strain is represented as the mean ± SD of seven measurements and the data from four individual transformants are shown for other strains.

transformed strains was assessed in YES medium, because many of strains co-expressing CoQ biosynthetic genes reduced growth rate when minimal medium was used. The CoQ_{10} productivity of the wild type strain did not change significantly in YES medium (0.5–0.6 μg/ml, 0.4–0.5 mg/g-DCW) and minimal medium (0.6–0.7 μg/ ml, 0.4–0.5 mg/g-DCW). In most cases, no significant increases in production were observed when two genes were coexpressed; however, CoQ_{10} production per cell (but not per volume or DCW) increased twofold, compared with wild type, in strains expressing *ppt1* and *coq5* (Fig. 2-5). I next coexpressed four genes simultaneously and obtained twofold increases in CoQ_{10} production per cell (but not per volume or DCW) when *ppt1*, *coq3*, *coq5*, and *coq6* were simultaneously expressed. These increases are mainly due to changes of the cell mass since I did not see significant increases of CoQ_{10} production per DCW. No production increases were seen for other gene combinations. Six, eight, and ten genes were also coexpressed, but no significant increases were seen in CoQ_{10} production.

Manipulation of genes upstream of CoQ biosynthesis

The overexpression of CoQ biosynthesis genes was only minimally successful in enhancing CoQ production. Therefore, I next attempted to improve CoQ_{10} productivity by increasing the supply of the CoQ_{10} precursors PHB and decaprenyl diphosphate (DPP). Nine different biosynthetic genes from the shikimate and mevalonate pathways were overexpressed in *S. pombe* (Fig. 2-6). The genes encoded the following proteins: truncated HMG-CoA reductase from *S. cerevisiae* that had no inhibitory regulation in the mevalonate pathway (*Sce_thmgr1*) (Donald et al. 1997), feedback-inhibition-resistant mevalonate kinase from *S. cerevisiae* (*Sce_mvk*^{FBR}) (Bai et al. 2011), phosphomevalonate kinase from *S. pombe* (*Spo_erg8*, SPAC343.01c), diphosphomevalonate decarboxylase from *S. pombe* (*Spo_mvd1*,

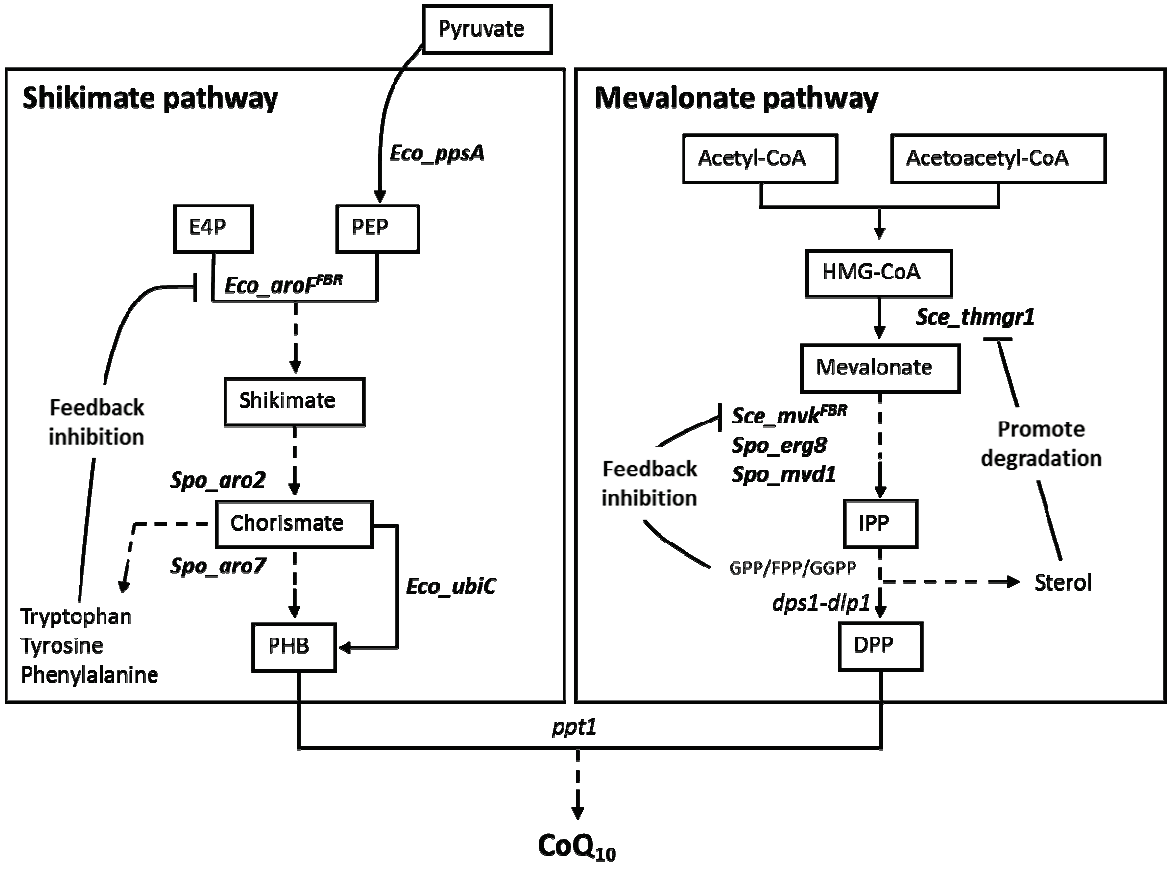

Fig. 2-6 Shikimate and mevalonate pathways upstream of CoQ biosynthesis

The shikimate pathway that leads to the synthesis of PHB and the mevalonate pathway that leads to the synthesis of DPP are indicated. *Eco*, *E. coli* genes; *Spo*, *S. pombe* genes; *Sce*, *S. cerevisiae* genes. *Eco_ppsA*, phosphoenolpyruvate synthase; *Eco_aroF*^{FBR}, feedback-inhibitionresistant (FBR) 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase; *Eco_ubiC*, chorismate lyase; *Spo_aro2*, chorismate synthase; *Spo_aro7*, chorismate mutase; *Sce_thmgr1*, truncated 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase 1; *Sce_mvk*^{FBR}, FBR mevalonate kinase; *Spo_erg8*, phosphomevalonate kinase; *Spo_mvd1*, diphosphomevalonate decarboxylase; *dps1-dlp1*, decaprenyl diphosphate synthase; *ppt1*, PHB-decaprenyl diphosphate transferase.

SPAC24C9.03), feedback-inhibition-resistant DAHP synthase from *E. coli* (*Eco_aroF*^{FBR}) (Weaver et al. 1990), chorismate synthase from *S. pombe* (*Spo_aro2*, SPCC1223.14), chorismate mutase from *S. pombe* (*Spo_aro7*, SPAC16E8.04c), phosphoenolpyruvate synthase from *E. coli* (*Eco_ppsA*), and chorismate lyase from *E. coli* (*Eco_ubiC*). Plasmids were constructed by inserting the appropriate PCR amplicon into pREP1. Plasmids were introduced into wild type *S. pombe* and CoQ_{10} production was assessed. As shown in Fig. 2-7, overexpression of *Eco_ubiC* or *Sce_thmgr1* gene resulted in a CoQ_{10} productivity increase of approximately 30%. Overexpression of the *Eco_aroF*^{FBR} gene increased productivity by approximately 15%. By contrast, when the *Spo_aro7* gene was overexpressed, a significant decrease in CoQ_{10} productivity was observed as a result of the accompanying reduced growth rate. These results indicated that, in some cases, overexpression of genes upstream of the CoQ_{10} pathway led to CoQ_{10} productivity improvements in *S. pombe*.

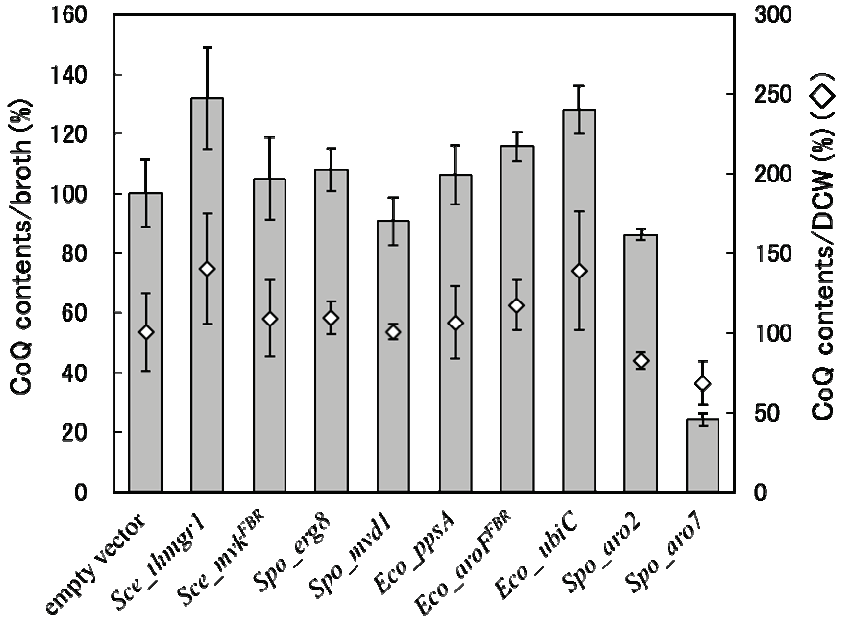

Fig. 2-7 Enhancement of CoQ_{10} production by overexpression of shikimate and mevalonate pathway genes

CoQ_{10} productivity of strains expressing a single gene located upstream of CoQ biosynthesis was compared to reference strain CHP429 (empty vector). Strains were grown at 30˚C in PM minimal medium containing adenine, uracil, and histidine. Cultures were initiated at 1×10^5 cells/ml and harvested after 48 h growth. Production of CoQ_{10} was measured by HPLC. *Gray bars* represent relative CoQ_{10} content per volume (%) and *open diamonds* represent relative CoQ_{10} content per dry cell weight (DCW) (%). Data are represented as the mean ± SD of three measurements.

Simultaneous overexpression of genes involved in dual pathways

Three of the upstream genes that produced improvements in CoQ_{10} productivity were coexpressed and CoQ_{10} productivity was evaluated. *S. pombe* CHP429 was transformed with plasmids pREP1-Sce_thmgr1 and pREP2-Eco_aroF^{FBR}, pREP1-Sce_thmgr1 and pREP2-Eco_ubiC, pREP1-Eco_aroF^{FBR} and pREP2-Eco_ubiC, or pREP1-Sce_thmgr1, pREP2-Eco_aroF^{FBR}, and pREP1-HIS7-Ec_ubiC. CoQ_{10} production was assessed (Fig. 2-8) and a twofold increase was observed in the strains harboring pREP1-Sce_thmgr1 and pREP2-Eco_aroF^{FBR}, and pREP1-Sce_thmgr1 and pREP2-Eco_ubiC. No further gain in productivity was observed when all three plasmids (pREP1-Sce_thmgr1, pREP2-Eco_aroF^{FBR}, and pREP1-HIS7-Ec_ubiC) were coexpressed. In this study, the coexpression of *S. cerevisiae thmgr1* and *E. coli ubiC* was the most effective combination for increasing CoQ_{10} productivity.

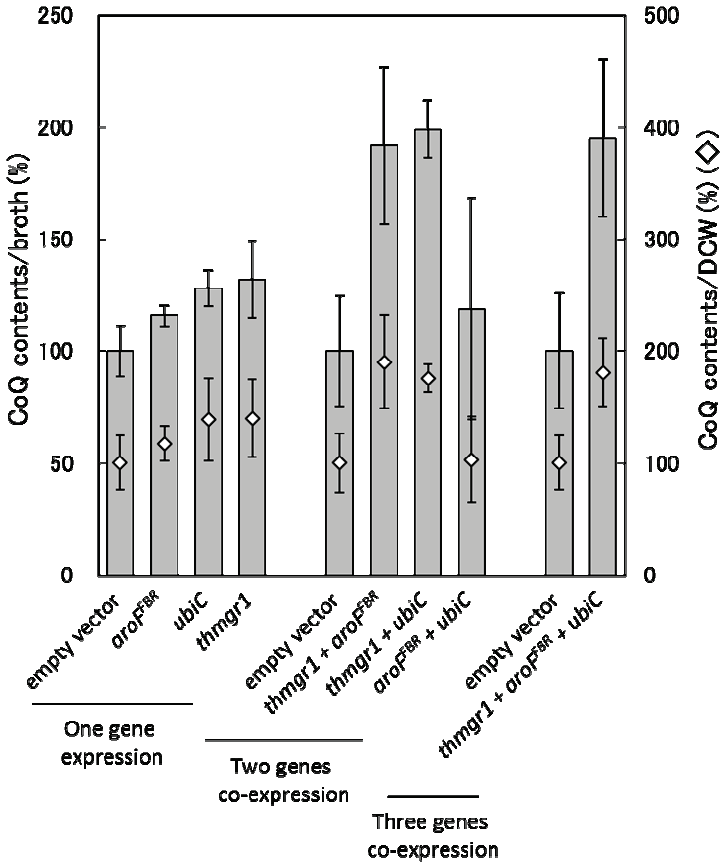

Fig. 2-8 Effect of coexpression of multiple upstream genes on CoQ_{10} production

CoQ_{10} production was compared between strains co-expressing two or three genes and reference strain CHP429 (empty vector). Strains were grown at 30˚C in PM minimal medium containing adenine and histidine, or adenine. Cultures were initiated at 1×10^5 cells/ml and harvested after 48 h growth. Production of CoQ_{10} was measured by HPLC. *Gray bars* represent relative CoQ_{10} content per volume (%) and *open diamonds* represent relative CoQ_{10} content per DCW (%). Data are represented as the mean ± SD of three measurements.

Discussion

In this study, I aimed to increase CoQ_{10} production in *S. pombe* by overexpression of various CoQ_{10} biosynthetic genes. *S. pombe* is a widely used research eukaryote alongside *S. cerevisiae*. It produces CoQ_{10} naturally, and much is already understood regarding its CoQ_{10} biosynthesis (Uchida et al. 2000; Hayashi et al. 2014; Saiki et al. 2003a; Saiki et al. 2003b); thus, it was a promising organism to use with this approach.

Industrial-scale bio-production of CoQ_{10} by microorganisms has been established by several companies. Microorganisms that produce CoQ_{10}, including photosynthetic bacteria and yeasts, were selected for its high yield and used for the fermentation production. Until now, successful approaches for the production of CoQ_{10} have relied predominantly on bacterial or yeast mutants selected for their high CoQ_{10} content. However, in recent years, an application of genetic engineering to produce CoQ_{10} was attempted in *E. coli* (Park et al. 2005) and the production of CoQ_{10} reached to 22.5 mg/l. In this case, DPP synthase from *Gluconobacter suboxydans* needs to be expressed. On the other hand, *A. tumefaciens* and *R. sphaeroides* have been used to produce CoQ_{10} and reached to 548.2 and 93.3 mg/l productivity, by engineering the MEP pathway (Yoo et al 2008; Lu et al. 2014). These successful results in bacteria stimulated my approaches to improve CoQ_{10} productivity in *S. pombe* by genetic engineering.

Overexpression of the genes directly involved in CoQ_{10} biosynthesis (*dps1-dlp1*, *ppt1*, or *coq3-coq9*) led to only minimal increases in CoQ_{10} productivity in *S. pombe*. One possible reason for this failure was that overexpression of the *coq* genes caused severe growth inhibition (Fig. 2-3). High expression of proteins localized to the mitochondria tends to inhibit cell growth, and this might then undermine any beneficial effect of overexpression. Alternatively, individual proteins in the CoQ_{10} biosynthetic pathway might not be rate-limiting, and, therefore, expression of one or several genes may not be sufficient to produce an overall increase in productivity. Similarly, CoQ biosynthetic enzymes in *S. pombe* may cooperate within a large complex, as is the case in *S. cerevisiae* (He et al. 2014). I therefore simultaneously enhanced the expression of multiple CoQ biosynthetic genes; however, even when the 10 known CoQ biosynthetic genes were simultaneously overexpressed, CoQ_{10} productivity remained largely unimproved.

Some strains showed twofold increases in CoQ_{10} production per cell when *ppt1* and *coq5*, or *ppt1*, *coq3*, *coq5*, and *coq6*, were simultaneously expressed, but the changes were not observed in CoQ_{10} production per volume or per DCW. These cultures included many elongated cells, and I therefore considered that the higher CoQ production per cell might have

been due to the decrease in cell numbers resulting from delayed cell division. Such an effect might be caused by insertion of the transforming DNA into chromosomal sequences responsible for cell division.

A 3.7-fold improvement in CoQ_{10} production was previously achieved by expression of the *ppt1* gene in *S. pombe* (Zhang et al. 2007a); however, I was unable to repeat this result in this study and severe growth inhibition was observed when *ppt1* was overexpressed. Differences in the CoQ productivity of the control strains between the two studies may explain this discrepancy. CoQ productivity in control strains was constant regardless of the passage of time in the previous experiment (Zhang et al. 2007a), but in my study CoQ expression increased over time. These differences in control CoQ production would explain the calculated difference in the production of CoQ_{10} in cells expressing *ppt1*.

I next overexpressed genes encoding CoQ_{10} biosynthetic precursors (*Sce_thmgr1*, *Eco_aroF*^{FBR}, and *Eco_ubiC*). Individual overexpression of these genes led to improved production of CoQ, and simultaneous coexpression yielded further gains. These genes were previously reported to improve CoQ productivity in *E. coli* (*Eco_aroF*^{FBR} and *Eco_ubiC*) (Zhang et al. 2007b; Cluis et al. 2011) and *S. pombe* (HMG-CoA reductase from *S. pombe*) (Cheng et al. 2010). *Eco_aroF*^{FBR} and *Eco_ubiC* were previously examined only in *E. coli*, and this is the first report of their effectiveness in fission yeast.

Overexpression of the HMG-CoA reductase gene from *S. pombe* prompted a 2.7-fold increase in CoQ production in a previous study (Cheng et al. 2010). A truncated HMG-CoA reductase from *S. cerevisiae* was used in the present study. The difference in CoQ productivity between the studies might therefore be explained by the differences in the gene used, host organism, and/or culture conditions.

The results presented in this study indicate that the overproduction of precursors to the CoQ biosynthetic pathway is an effective strategy for improving CoQ productivity. As the effective genes *Sce_thmgr1*, *Eco_aroF*^{FBR}, and *Eco_ubiC* were from organisms other than *S. pombe*, I speculate that tight regulation was lost in *S. pombe* upon transformation with these sequences and that this led to the observed increases in CoQ_{10} production.

In conclusion, overexpression of *dps1-dlp1* or *coq3* in *S. pombe* resulted in a slight (but not significant) increase in CoQ_{10} production. Overexpression of all ten *coq* genes did not produce an increase in CoQ_{10} productivity; however, overproduction of upstream biosynthetic precursors through the expression of genes such as *Sce_thmgr1*, *Eco_aroF*^{FBR}, and *Eco_ubiC* improved CoQ_{10} production in *S. pombe* twofold.

Chapter 3

Cloning and characterization of decaprenyl diphosphate synthase from three different fungi

Abstract

Coenzyme Q (CoQ) is composed of a benzoquinone moiety and an isoprenoid side chain of varying lengths. The length of the side chain is controlled by polyprenyl diphosphate synthase. In this study, *dps1* genes encoding decaprenyl diphosphate synthase were cloned from three fungi: *Bulleromyces albus*, *Saitoella complicata*, and *Rhodotorula minuta*. The predicted Dps1 proteins contained seven conserved domains found in typical polyprenyl diphosphate synthases and were 528, 440, and 537 amino acids in length in *B. albus*, *S. complicata*, and *R. minuta*, respectively. *Escherichia coli* expressing the fungal *dps1* genes produced CoQ_{10} in addition to endogenous CoQ_8. Two of the three fungal *dps1* genes (from *S. complicata* and *R. minuta*) were able to replace the function of *ispB* in an *E. coli* mutant strain. *In vitro* enzymatic activities were also detected in recombinant strains. The three *dps1* genes were able to complement a *Schizosaccharomyces pombe dps1*, *dlp1* double mutant. Recombinant *S. pombe* produced mainly CoQ_{10}, indicating that the introduced genes were independently functional and did not require *dlp1*. The cloning of *dps1* genes from various fungi has the potential to enhance production of CoQ_{10} in other organisms.

Introduction

CoQ (coenzyme Q or ubiquinone), a natural compound present in almost all living organisms, localizes primarily to the plasma membrane in prokaryotes and to the mitochondrial inner membrane in eukaryotes. CoQ is essential for aerobic growth and oxidative phosphorylation in the electron transport system (Vinothkumar et al. 2014), antioxidation (Quinzii et al. 2014), disulfide formation (Bader et al. 1999), sulfide oxidation (Saiki et al. 2003b), and *de novo* UMP synthesis (López-Martín et al. 2007; Matsuo et al. 2013). The biochemical properties and the ongoing discovery of novel functions of CoQ have prompted substantial research interest. For example, one area of research focused on the role of human-type CoQ (CoQ_{10}) in cardiovascular disease and its use in clinical therapies and nutrition (Ayer et al. 2015).

CoQ is composed of a benzoquinone moiety and an isoprenoid side chain of varying lengths. Although the CoQ biosynthetic pathway in *Escherichia coli* has been almost entirely characterized, additional factors remain to be determined in eukaryotes (Allan et al. 2015; Aussel et al. 2014; Kawamukai 2016). In *E. coli*, generation of the isoprenoid side chain is catalyzed by polyprenyl diphosphate synthase (poly-PDS). The isoprenoid side chain is

condensed with *p*-hydroxybenzoate (PHB) or *p*-aminobenzoic acid (pABA) (in *Saccharomyces cerevisiae*) by PHB-polyprenyl diphosphate transferase (Fig. 3-1). A series of modification reactions of the benzoquinone ring, including methylations, decarboxylation, and hydroxylations, completes CoQ processing (Kawamukai 2016). CoQ biosynthetic genes in complex eukaryotes are thought to be similar to those found in *S. cerevisiae*, with the exception of those involved in isoprenoid side chain synthesis (Kawamukai 2009).

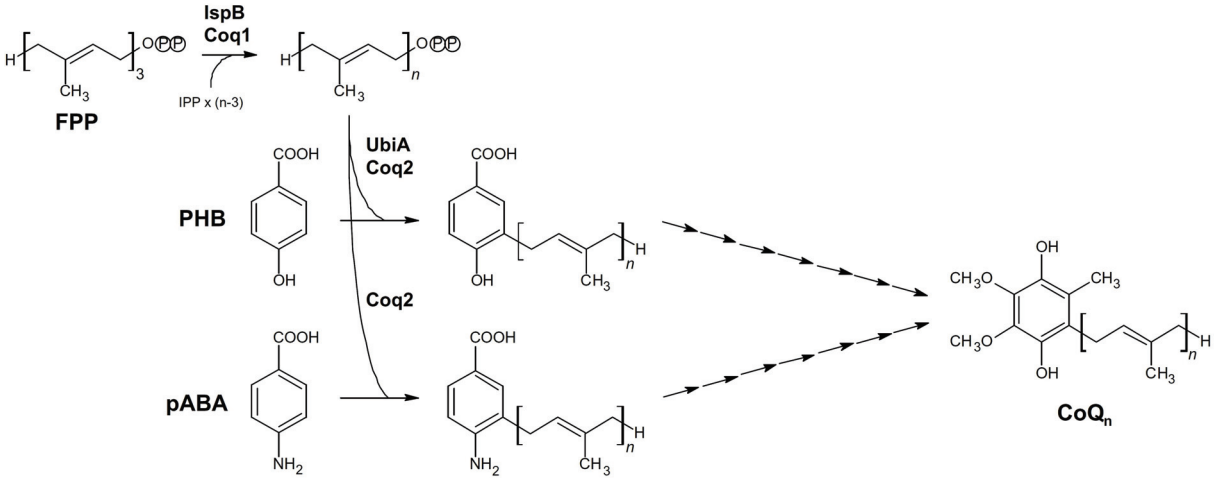

Fig. 3-1 Proposed coenzyme Q (CoQ) biosynthetic pathway
The biosynthetic pathway that converts PHB into CoQ consists of eight steps in prokaryotes (represented by *E. coli*) and eukaryotes (represented by *S. cerevisiae*). Starting from the condensation of PHB with trans-polyprenyl diphosphate, seven modifications of the aromatic ring are then needed to produce CoQ. In *S. cerevisiae*, pABA is also used as a substrate in addition to PHB. IspB (in *E. coli*) and Coq1 (in *S. cerevisiae*) convert FPP (farnesyl diphosphate) into PPP (polyprenyl diphosphate). UbiA (in *E. coli*) and Coq2 (in *S. cerevisiae*) catalyze the condensation of PHB and PPP.

CoQ side chain length varies between species. For example, *S. cerevisiae* has six isoprene units in its side chain, *Candida utilis* has seven units, *E. coli* has eight units, mice and *Arabidopsis thaliana* have nine units, and *Schizosaccharomyces pombe* and humans have ten units (Kawamukai 2002; Okada et al. 2004; Saiki et al. 2005; Zhu et al. 1995). The isoprenoid side chain length of CoQ is defined by the product generated by poly-PDS (Okada et al. 1998a; Okada et al. 1996; Suzuki et al. 1994), but not by the substrate specificity of PHB-polyprenyl diphosphate transferases (Okada et al. 2004; Suzuki et al. 1994). *E. coli* ordinarily produces CoQ_8, but exogenous expression of heptaprenyl, solanesyl, or decaprenyl diphosphate synthase (deca-PDS) genes from *Haemophilus influenzae*, *Rhodobacter capsulatus*, or *Gluconobacter suboxydans*, respectively, allows production of CoQ_7, CoQ_9, or

CoQ_{10}, respectively (Okada et al. 1998b; Okada et al. 1997a; Okada et al. 1997b; Park et al. 2005). Similarly, an *S. cerevisiae COQ1* disruptant that expressed various poly-PDS genes from different organisms produced the CoQ type of the donor organism (e.g., CoQ_5, CoQ_6, CoQ_7, CoQ_8, CoQ_9, or CoQ_{10}) (Okada et al. 1998a). Furthermore, when genetic engineering was used to enable deca-PDS production by rice mitochondria, the rice produced CoQ_{10} instead of the originally synthesized CoQ_9 (Takahashi et al. 2006). Long-chain PDSs for the production of CoQ side chains were previously characterized in many organisms and these are classified into the chain length they produce, sources of species, and compositions of proteins (Table 3-1).

Table 3-1 Classification of polyprenyl diphosphate synthases

Species	Structure	Products	References
Saccharomyces cerevisiae	Homomer	C_{30}	Ashby and Edwards 1990; Ayer et al. 2015; Zhang et al. 2008
Sulfolobus solfataricus	Homodimer	C_{30}	Sun et al. 2005
Escherichia coli	Homodimer	C_{40}	Asai et al. 1994; Kainou et al. 2001
Plasmodium falciparum	Homomer	C_{40}	Tonhosolo et al. 2005
Arabidopsis thaliana	Homodimer	C_{45}	Hirooka et al. 2003; Jun et al. 2004
Rhodobacter capsulatus	Homodimer	C_{45}	Okada et al. 1997a
Trypanosoma cruzi	Homodimer	C_{45}	Ferella et al. 2006
Gluconobacter suboxydans	Homodimer	C_{50}	Okada et al. 1998b; Lee et al. 2004
Mycobacterium tuberculosis	Homodimer	C_{50}	Chan et al. 2014
Rhodobacter sphaeroides	Homodimer	C_{50}	Seo et al. 2006; Zahiri et al. 2006
Sinorhizobium meliloti	Homomer	C_{50}	Cluis et al. 2007
Micrococcus luteus	Heterodimer	C_{30}	Shimizu et al. 1998
Mus musculus	Heterotetramer	C_{45}	Saiki et al. 2005
Aphis gossypii	Heteromer	C_{50}	Zhang and Li, 2013
Homo sapiens	Heterotetramer	C_{50}	Saiki et al. 2005
Schizosaccharomyces pombe	Heterotetramer	C_{50}	Saiki et al. 2003a; Suzuki et al. 1997

Long-chain PDSs are classified into homodimer (e.g., *IspB* in *E. coli*) and heterotetramer (e.g., Dps1 and Dlp1 in *S. pombe*) types based on the pattern of their components. H omodimeric enzymes and Dps1 contain seven conserved regions (domains I–VII) and two aspartate-rich motifs DDXXD (domains II and VI). The first DDXXD motif is responsible for binding with FPP, and the second is responsible for binding with IPP. The Dlp1 protein is weakly similar in the sequence to Dps1 but lacks the conserved regions of domains II and VI (Saiki et al. 2003a). Solanesyl- and deca-PDSs from mice and humans were found to be heterotetramer types (Saiki et al. 2005).

To date, only heteromeric deca-PDSs have been identified in eukaryotes. Therefore, cloning and expression of eukaryotic homomeric deca-PDS may be desirable for the production of CoQ_{10} in various species. In this study, decaprenyl diphosphate synthase (*dps1*) genes were cloned from the fungi *Saitoella complicata*, *Bulleromyces albus*, and *Rhodotorula minuta* and characterized in *E. coli* and *S. pombe*.

Materials and methods

Materials

Restriction enzymes and other DNA-modifying enzymes were purchased from Takara Shuzo Co., Ltd., and New England Biolabs, Inc. IPP, (*E*)-farnesyl diphosphate (all-(*E*)-FPP), geranylgeraniol, and solanesol (all-(*E*)-nonaprenol) were purchased from Sigma. [1-^{14}C]IPP (1.96 TBq/mol) was purchased from GE Healthcare UK Ltd. Kieselgel 60 F_{254} TLC plates were purchased from Merck. Reversed-phase LKC-18 TLC plates were purchased from Whatman.

Strains, media, and plasmids

Fungal strains *Saitoella complicata* NBRC 10748, *Bulleromyces albus* NBRC 1192, and *Rhodotorula minuta* NBRC 0387 were purchased from the NITE Biological Resource Center (NBRC). Fungi were grown in YM medium (1% glucose, 0.5% peptone, 0.3% yeast extract, and 0.3% malt extract). *E. coli* strain DH5α was used for general plasmid construction and gene expression. KO229 (Cm^r, Sp^r, *ispB::cat*), an *ispB*-defective mutant of *E. coli*, harboring plasmid pKA3 (*ispB*) (Okada et al. 1997b), was used as a host strain to express fungal *dps1* genes for CoQ synthesis and complementation analysis. *E. coli* strains were cultured in LB medium (1% bactotryptone, 0.5% yeast extract, and 1% NaCl; pH 7.0). Plasmids pT7Blue T-Vector (Novagen, Madison, WI, USA) and pUCNT were used as vectors. pUCNT, which contained an *Nde*I restriction site, was modified from pUC19 (Ikenaka et al. 2003). *S. pombe* wild-type strain PR110 (Saiki et al. 2005) and a double-deletion mutant of *dps1* and *dlp1*, LA1 (*Δdlp1::ura4::ADE2*, *Δdps1::kanMx6*) (Zhang et al. 2008a), were used to express fungal *dps1* genes for CoQ synthesis and complementation analysis. *S. pombe* strains were grown in PM medium (0.3% potassium hydrogen phthalate, 0.56% sodium phosphate, 0.5% ammonium chloride, 2% glucose, vitamins, minerals, and salts). Plasmid pREP1 was used as the vector.

Cloning of dps1 from B. albus

Genomic DNA was prepared from *B. albus* as described previously (Hoffman and Winston 1987). Total RNA was prepared from *B. albus* using an RNeasy mini kit (Qiagen, Valencia, CA), and mRNA was isolated using an Oligotex-dT30 < Super > mRNA purification kit (Takara Shuzo Co., Ltd.). Primers DPS-1 and DPS-1 1AS (Table 3-2) were used for PCR amplification, and the resulting 220 bp amplicon was cloned into pT7Blue T-Vector to yield pT7-B1DPS. The inserted amplicon was sequenced. To obtain the 3' region of the *dps1* gene, RT-PCR and PCR were performed using a PrimeScript High Fidelity RT-PCR Kit (Takara Shuzo Co., Ltd.), with primer B1S, whose sequence was in pT7-B1DPS, and mRNA as a template. An 850 bp fragment was obtained and cloned into pT7Blue T-Vector to yield pT7-B2DPS. To obtain the 5' region of the *dps1* gene, RT-PCR was performed using a 5'-Full RACE Core Set (Takara Shuzo Co., Ltd.), with primer B7ASP (phosphorylated at the 5' end), whose sequence was in pT7-B2DPS, and mRNA as a template. The resultant amplified cDNA fragment was circularized and used a template for PCR with primers B5S and B4AS, whose sequences were in pT7-B2DPS. The resultant amplified fragment was used as a template for PCR with nested primers B6S and B3AS. The resultant 950 bp fragment was cloned into pT7Blue T-Vector to yield pT7-B3DPS. To obtain the full-length *dps1* gene, the 5' region was amplified using BN1 and B3AS primers with pT7-B3DPS as a template, and the 3' region was amplified using B1S and BCH primers with pT7-B2DPS as a template. The resulting fragments (910 and 750 bp) were mixed and the full-length duplex was formed by denaturation, annealing, and extension with DNA polymerase. The full-length *dps1* gene was then amplified using BN1 and BCH primers. The 1.6 kb DNA fragment was digested with *Nde*I and *Hin*dIII and cloned into similarly digested pUCNT to yield plasmid pNTB1-1. pREP1-B1dps1 was constructed using the gap-repair cloning (GRC) method (Matsuo et al. 2010). A fragment containing the *dps1* gene and flanking *nmt1* promoter and terminator sequences was amplified from pNTB1-1 using primers GRC_B1dps1-Fw and GRC_B1dps1-Rv. *S. pombe* PR110 was co-transformed with the *dps1* gene and a *Bam*HI/*Sma*I-digested pREP1 vector. Transformants were screened with PM plates containing uracil and pREP1-B1dps1 was extracted.

Cloning of dps1 from S. complicata

Genomic DNAwas prepared from *S. complicata* as described previously (Hoffman and Winston 1987). Primers DPS-1 and DPS-1 1AS were used for PCR amplification, and the

resulting 220 bp amplicon was cloned into pT7Blue T-Vector to yield pT7-SaDPS. The inserted amplicon was sequenced. *S. complicata* genomic DNA was digested with *Eco*RI, and Southern analysis was performed using standard methods (Sambrook et al. 1989). Southern blots were probed with a 145 bp DNA fragment amplified using primers Sa-1S and Sa-2AS and labeled with an ECL direct nucleic acid labeling and detection system (GE Healthcare). Hybridization and detection of the labeled probe were performed according to the manufacturer's instructions. Southern analysis identified a 10 kb fragment, which was then excised from the gel and packaged using a Lambda DASH II phage kit (Agilent technology). Phage libraries were propagated in *E. coli* strain XL1-Blue MRA (P2). Plaque hybridization was performed using the same probe, and six positive phages were isolated. DNA from positive phage libraries was digested with *Sal*I or *Sac*I and electrophoresed. Southern analysis of the digested DNA identified 4.5 and 3.5 kb genomic fragments in the *Sal*I and *Sac*I digested DNA, respectively. Another 3.0 kb fragment was sequenced for the full length of dps1. The full-length *S. complicata dps1* gene was amplified using primers Sa-N1 and Sa-C, digested with *Nde*I and *Eco*RI, and cloned into similarly digested pUCNT to yield pNTSa1-1. pREP1-Sa1dps1 was constructed using the GRC method. The *dps1* gene was amplified from pNTSa1-1 using primers GRC_Sa1dps1-Fw and GRC_Sa1dps1-Rv. *S. pombe* PR110 was then co-transformed with the *dps1* gene and a *Bam*HI/*Sma*I-digested pREP1 vector. Transformants were screened on PM plates containing uracil, and pREP1-Sa1dps1 was extracted.

Cloning of dps1 from R. minuta

Genomic DNA was prepared from *R. minuta* as described previously (Hoffman and Winston 1987). Primers DPS-1 and DPS-1 1AS were used for PCR amplification, and the resulting 220 bp amplicon was cloned into pT7Blue T-Vector to yield pT7-RmDPS. The inserted amplicon was sequenced. *R. minuta* genomic DNA was digested with *Eco*RI, and Southern analysis was performed using standard methods (Sambrook et al. 1989). Southern blots were probed with a 144 bp DNA fragment amplified using primers Rm-1S and Rm-2AS, and labeled with an ECL direct nucleic acid labeling and detection system (GE Healthcare). Hybridization and detection of the labeled probe were performed according to the manufacturer's instructions. Southern analysis identified a 5.5 kb fragment, which was then excised from the gel and packaged using a Lambda DASH II phage kit (Agilent technology). Phage libraries were propagated in *E. coli* strain XL1-Blue MRF'. Plaque hybridization was

performed using the same probe, and seven positive phages were isolated. Positive phagemid DNA was prepared by *in vivo* excision using the Lambda DASH II phage kit (Agilent technology). Approximately 1.6 kb of insert was sequenced, and this contained the full-length *dps1* gene. The full-length *R. minuta dps1* gene was amplified using primers RM-1 and Rm-CE2, digested using *Nde*I and *Eco*RI, and then cloned into similarly-digested pUCNT to yield pNTRm2-1. For construction of shortened *R. minuta dps1*, a 1.2 kb fragment was amplified using primers Rm-4 and Rm-CE2 and digested with *Nde*I and *Nhe*I to produce a 600 bp fragment. This shortened fragment was used to replace the *Nde*I and *Nhe*I fragment (1.0 kb) in pNTRm2-1 to yield pNTRmSsp. A 70 bp fragment was amplified using primers RM-1 and RM-6R, digested with *Nde*I and *Ssp*I, and cloned into similarly-digested pNTRmSsp to yield pNTRm6-1. pREP1-Rm2dps1 and pREP1-Rm6dps1 were constructed using the GRC method. The *dps1* genes were amplified from pNTRm2-1 and pNTRm6-1 using primers GRC_Rmdps1-Fw and GRC_Rmdps1-Rv. *S. pombe* PR110 was then co-transformed with the *dps1* gene and a *Bam*HI/*Sma*I-digested pREP1 vector. Transformants were screened on PM plates containing uracil, and then pREP1-Rm2dps1 and pREP1-Rm6dps1 were extracted.

Table 3-2 Primers used in this study

DPS-1	5′-AAGGATCCTNYTNCAYGAYGAYGT-3′
DPS-1 1AS	5′-ARYTGNADRAAYTCNCC-3′
B1S	5′-TCGGCATTACGGCGGAACCTG-3′
B7ASP	5′-CACACCATCAGACTC-3′
B5S	5′-TGGGTCAAGGATGTGGCGTA-3′
B4AS	5′-CAGATTGGCGATGACCGTCGC-3′
B6S	5′-GTTCGCCTACAAGCAGAACCC-3′
B3AS	5′-TTGAGCCAAAGGCGGATGGG-3′
BN1	5′-AAGGATCCATATGTTTCGTTCGGCGCGG-3′
BCH	5′-CCAAGCTTCTACTTCACTCTTCCAC-3′
Sa-1S	5′-GAGACCAGACGAAACGCACCA-3′
Sa-2AS	5′-TAACAGTAGCCAAAAGCTCAATCA-3′
Sa-N1	5′-AACATATGGCCTCACCAGCACTGCGG-3′
Sa-C	5′-AAGAATTCCTATCTTGACCTAGTCAACAC-3′
Rm-1S	5′-GCCATGAGGAGAGCACAAGCG-3′
Rm-2AS	5′-CACGGAGGCTACTAGCTCGAC-3′
RM-1	5′-ATCATATGATGCACCGACAAGCT-3′
Rm-CE2	5′-AAGAATTCCTACTTTGTTCGGTTGAGCACAG-3′
Rm-4	5′-ATCATATGAATATTCGACCCACTCCAACT-3′
RM-6R	5′-ACAATATTGTATTGAGGGCATTCGGGCGACTGC-3′
GRC_B1dps1-Fw	5′-GACTTATAGTCGCTTTGTTAAATCATATGTCGACTCTAGAGGATCCAATGTTTCGTTCGG-3′
GRC_B1dps1-Rv	5′-AAAAACCCTAGCAGTACTGGCAAGGGAGACATTCCTTTTACCCGGGCTACTTCACTCTTT-3′
GRC_Sa1dps1-Fw	5′-GACTTATAGTCGCTTTGTTAAATCATATGTCGACTCTAGAGGATCCAATGGCCTCACCAG-3′
GRC_Sa1dps1-Rv	5′-AAAAACCCTAGCAGTACTGGCAAGGGAGACATTCCTTTTACCCGGGCTATCTTGACCTAG-3′
GRC_Rmdps1-Fw	5′-GACTTATAGTCGCTTTGTTAAATCATATGTCGACTCTAGAGGATCCAATGATGCACCGAC-3′
GRC_Rmdps1-Rv	5′-CCTAGCAGTACTGGCAAGGGAGACATTCCTTTTACCCGGGCTACTTTGTTCGGTTGAGCA-3′

Complementation of ispB in an E. coli ispB disruption mutant with fungal dps1

E. coli KO229 (*ispB::cat*) harboring pKA3 (Okada et al. 1997b) was transformed with plasmid containing a fungal *dps1* gene to produce transformants resistant to spectinomycin and ampicillin. Transformants were subcultured five times in LB medium containing 50 μg/ml ampicillin and were plated on LB agar medium containing ampicillin. The resulting colonies were then replicated on LB medium containing ampicillin or spectinomycin. Transformants that were both spectinomycin-sensitive and ampicillin-resistant contained the fungal *dps1* plasmid but not pKA3 and were selected for further analysis.

CoQ extraction and analysis

Recombinant *E. coli* strains were incubated in LB liquid medium with appropriate antibiotics to the mid-to-late log phase, and then cells were collected by centrifugation at 3500 rpm. For yeast strains, minimum medium with appropriate supplements were used for incubation. CoQ was extracted as described previously (Okada et al. 1998b; Saiki et al. 2005). The crude CoQ extract was analyzed by normal-phase TLC with CoQ_{10} as the standard. Normal-phase TLC was carried out on a Kieselgel 60 F_{254} plate with benzene. The UV-visualized band containing CoQ was collected from the TLC plate and extracted with chloroform-methanol (1:1, vol/vol). The solution was evaporated to dryness and the residue was re-dissolved in ethanol. The purified CoQ was further analyzed by high-performance liquid chromatography (HPLC) using ethanol as a solvent. The CoQ_{10} quantification was performed by using CoQ_6 (Avanti Lipids Polar, Inc.) as an internal standard.

Prenyl diphosphate synthase assay and product analysis

PDS activity was assayed as described previously (Saiki et al. 2005). Cultures were grown to the mid-to-late log phase in the appropriate medium and then harvested by centrifugation. All subsequent steps were carried out at 4 °C. Cells were resuspended in a buffer containing 100 mM potassium phosphate (pH 7.4), 5 mM EDTA, and 1 mM 2-mercaptoethanol and ruptured by vigorous shaking with glass beads 14 times for 30 s at 60 s intervals on ice. The homogenate was centrifuged at 1500×g for 10 min, and the resulting supernatant was used as a crude enzyme extract. The incubation mixture contained 2 mM MgCl2, 0.2% (w/v) Triton X-100, 50 mM potassium phosphate buffer (pH 7.4), 5 mM KF, 10 mM iodoacetamide, 20 μM [1-^{14}C]IPP (specific activity 0.92 MBq mol^{-1}), 100 μM FPP, and 1.5 mg mL^{-1} of the enzyme in a final volume of 0.5 mL. Sample mixtures were incubated for 60 min at 30 °C.

Prenyl diphosphates were extracted with 1-butanol-saturated water and hydrolyzed with acid phosphatase. The hydrolysis products were extracted with hexane and analyzed by reverse-phase TLC with acetone/water (19:1, v/v). Radioactivity on the plate was detected with a BAS1500-Mac imaging analyzer (Fuji Film Co.). The spots of the marker prenols were visualized by exposure of the plate to iodine vapor.

Nucleotide sequence accession number

The sequences reported herein have been deposited in the GenBank database (*B. albus* Dps1, BD182059; *R. minuta* Dps1, BD170286; *S. complicata* Dps1, BD093645).

Results

Cloning of the fungal dps1 genes from B. albus, S. complicata, and R. minuta

Fungi that produced CoQ_{10} were identified in order to find target deca-PDS genes for cloning. CoQ species from three fungi, *B. albus*, *S. complicata*, and *R. minuta*, were examined, and all strains were found to produce CoQ_{10} (Fig. 3-2). The gene encoding deca-PDS was cloned from all three fungal species. PCR-mediated amplification methods were used to obtain fragments of the deca-PDS gene, which were then used to isolate the full-length gene.

Genomic DNAs were prepared from *B. albus*, *S. complicata*, and *R. minuta*. DNA fragments were amplified by PCR using primers DPS-1 and DPS-1 1AS, which corresponded to a conserved region in typical prenyl diphosphate synthases. DNA fragments of approximately 220 bp were obtained from each genomic DNA and cloned into the pT7BlueT-Vector. Fragments were sequenced, and translated amino acid sequences revealed a conserved motif, GDFLLXRA, known to be conserved in typical prenyl diphosphate synthases.

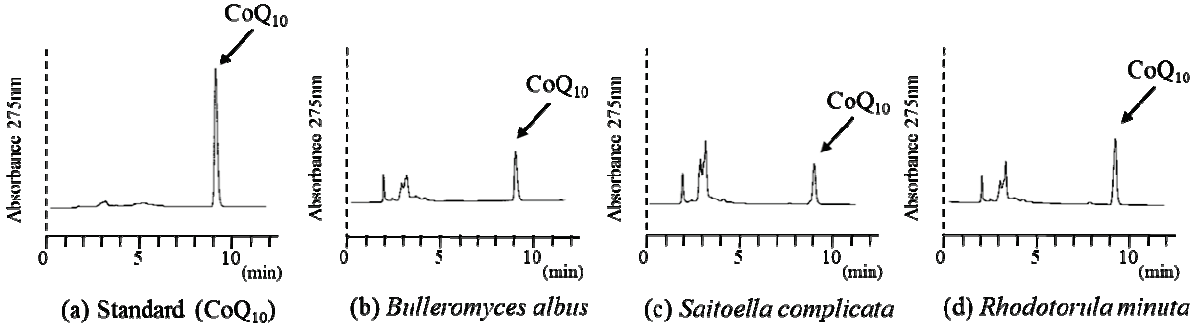

Fig. 3-2 Detection of CoQ species in three fungi

CoQ was extracted from three fungi and analyzed by high-performance liquid chromatography (HPLC). Standard CoQ_{10} (a) and CoQ extracted from *B. albus* (b), *S. complicata* (c), and *R. minuta* (d) are shown.

For *B. albus dps1* gene cloning, mRNA was prepared and used as template to amplify the 3' end of the cDNA by RT-PCR, because I predicted that *B. albus dps1* gene had introns based on the result of southern hybridization. Amplification used primer B1S, which corresponded to part of the 220 bp amplified genomic region, and produced a fragment of 850 bp. Sequence from the 3' cDNA was used to design primer B7ASP, which was used to amplify a 950 bp 5' region. Together, the 3' and 5' fragments constituted the full-length *dps1* cDNA from *B. albus*.

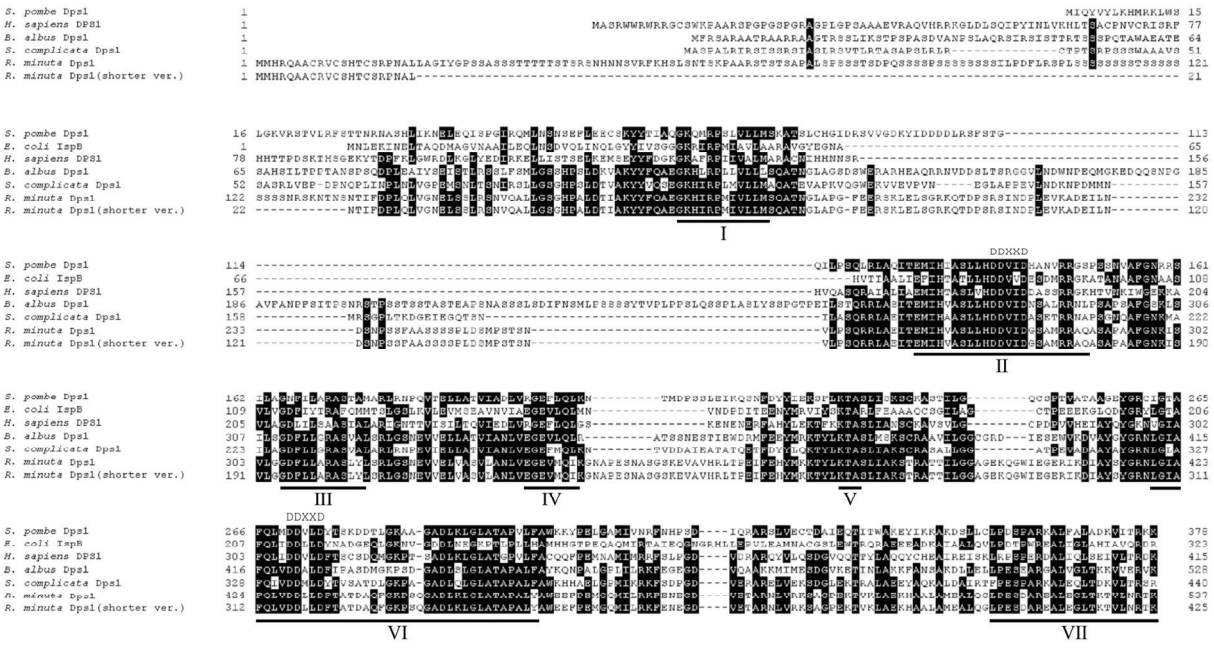

Fig. 3-3 Alignment of predicted amino acid sequences of deca-PDSs and octaprenyl diphosphate synthase

Amino acid residues that are identical in four or more sequences are indicated by *black boxes*. *Hyphens* indicate the absence of corresponding amino acid residues at those positions. Seven regions (I–VII) that are highly conserved in long-chain poly-PDSs are underlined. Two aspartate-rich motifs in domains II and VI, which are considered to be substrate binding sites in poly-PDSs, are denoted by "*DDXXD*". Sequences were aligned using GENETYX software. Genbank accession numbers as are follows: *S. pombe* Dps1, O43091; *Homo sapiens* DPS1, Q5T2R2; *E. coli* IspB, P0AD57; *B. albus* Dps1, BD182059; *R. minuta* Dps1, BD170286; *S. complicata* Dps1, BD093645.

A different approach was taken to clone deca-PDSs from *S. complicata* and *R. minuta*. The 220 bp fragments amplified from genomic DNA were used as probes for Southern hybridization of *Eco*RI-digested genomic DNA. DNA fragments of 10 and 5.5 kb were identified from *S. complicata* and *R. minuta*, respectively. The 10 and 5.5 kb genomic fragments were then extracted from the gel, cloned into phage DASH II, and packaged in

phage using an *in vitro* packaging kit. *E. coli* strains XL1-Blue MRA (P2) or MRF' were infected with phage and plated on NZY medium. Phage were transferred to N-bond filter, then denatured and neutralized. Nine filters were hybridized using the 220 bp probe, and a small number of plaques were positively identified. Positive phagemid DNAs were then sequenced to determine the full-length *dps1* sequence from *S. complicata* and *R. minuta*. Ultimately, *dps1* was successfully cloned from *B. albus*, *S. complicata*, and *R. minuta*.

Amino acid sequences were predicted from the *dps1* gene sequences (Fig. 3-3). The *B. albus*, *S. complicata*, and *R. minuta dps1* genes were predicted to encode 528, 440, and 537 amino acid proteins, respectively, and these exhibited 50, 51, and 46% sequence similarity to *S. pombe* Dps1. Alignment of other prenyl diphosphate synthases indicated that the predicted Dps1 proteins from *B. albus*, *S. complicata*, and *R. minuta* were long-chain prenyl diphosphate synthases.

Expression of fungal dps1 genes in E. coli

The *dps1* genes from *B. albus*, *S. complicata*, and *R. minuta* were expressed in wild-type *E. coli* strain DH5α, which naturally produces CoQ_8. CoQ species were tested from *E. coli* harboring pNTB1-1, which expressed *B. albus dps1*, pNTSa1-1, which expressed *S. complicata dps1*, pNTRm2-1, which expressed *R. minuta dps1*, or pNTRm6-1, which expressed a shorter version of *R. minuta dps1* (Fig. 3-4A). Because there is the serine-rich sequence, which does not align well with other Dps1s and might be a part of intron, in the N-terminal domain, a shorter version of Dps1 which lacks this region was made. Transformants all produced CoQ_{10}, albeit to varying degrees, in addition to CoQ_8 (Fig. 3-5). These results indicated that the cloned *dps1* genes encoded deca-PDSs and that they could be expressed in *E. coli*.

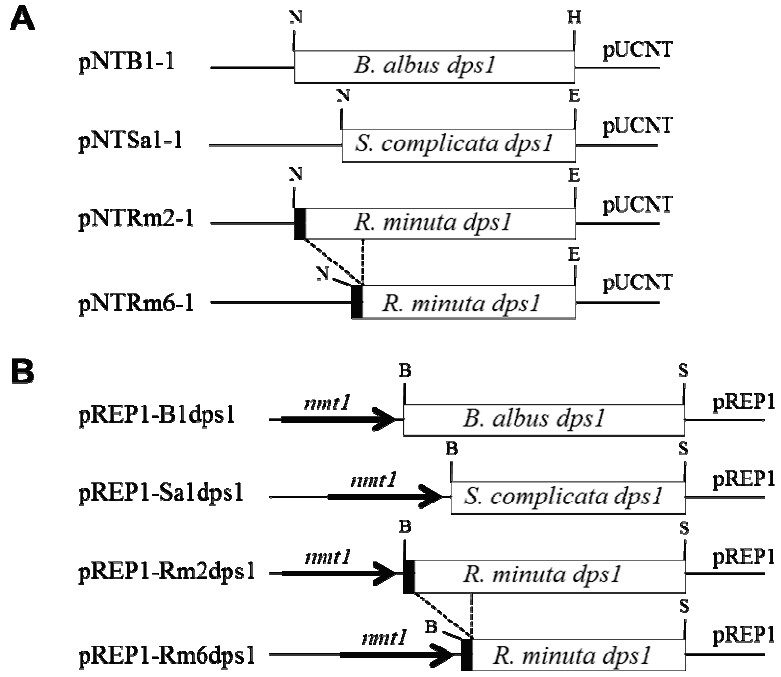

Fig. 3-4 Plasmid construction

A The cloning vector pUCNT, which contains an *Nde*I site, was modified from pUC19. Plasmids pNTB1-1, pNTSa1-1, and pNTRm2-1 contain full-length *dps1* genes from *B. albus*, *R. minuta*, and *S. complicata*, respectively, cloned into pUCNT. Plasmid pNTRm6-1 contains a truncated *R. minuta dps1* gene with a partial deletion in the 5′ region (see "Materials and Methods" section). **B** Genes encoding deca-PDS were inserted into the pREP1 vector under the control of the *nmt1* promoter for expression in *S. pombe*. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Nde*I; S, *Sma*I

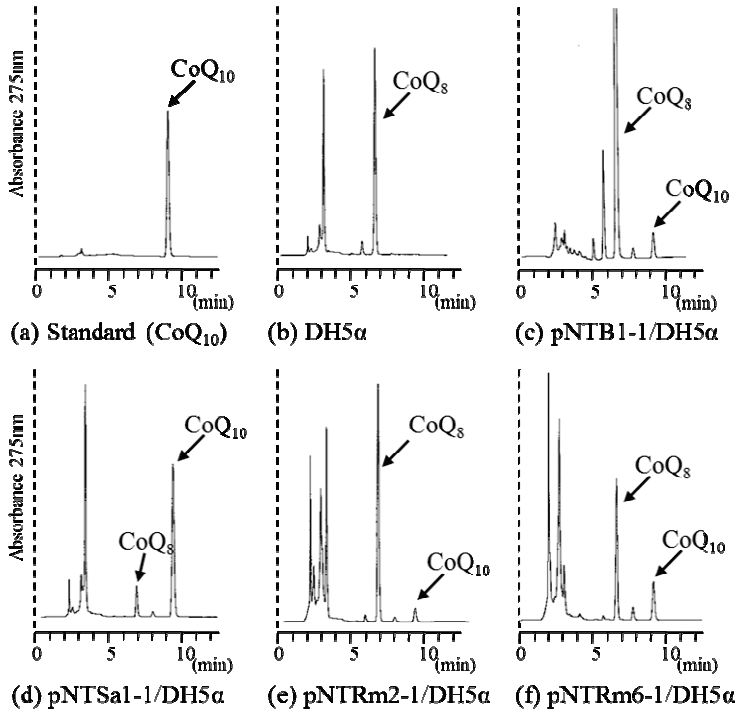

Fig. 3-5 Detection of CoQ in *E. coli* DH5α expressing fungal *dps1* genes

The *dps1* genes from *B. albus*, *S. complicata*, and *R. minuta* were expressed in *E. coli* DH5α. CoQ was extracted and analyzed using HPLC. Standard CoQ_{10} (a), and CoQ from DH5α (b), DH5α harboring pNTB1-1 (c), DH5α harboring pNTSa1-1 (d), DH5α harboring pNTRm2-1 (e), and DH5α harboring pNTRm6-1 (f) are shown.

Complementation of an E. coli ispB mutant by fungal dps1 genes

Long-chain prenyl diphosphate synthases are classified into two types: homomeric and heteromeric (Kawamukai 2009). Homomeric enzymes do not require additional subunits in order to be functional and would therefore be able to complement an *E. coli* mutant lacking the homologous gene (*ispB*). Complementation was examined by expressing fungal *dps1* genes in an *E. coli ispB* disruptant (KO229). Because *ispB* is essential for growth in *E. coli* (Okada et al. 1997b), KO229 harboring pKA3, which expresses *ispB*, was used. KO229 cells harboring both plasmids were grown for a few days in LB medium containing ampicillin. Cells containing pNTSa1-1 (or pNTRm2-1 or pNTRm6-1) but not pKA3 were isolated by selecting strains that were ampicillin-resistant and spectinomycin-sensitive. CoQ species from transformant strains were analyzed by HPLC (Fig. 3-6). *E. coli* KO229 harboring pKA3 synthesized only CoQ_8 (Fig. 3-6b), *E. coli* KO229 harboring pNTSa1-1 predominantly synthesized CoQ_{10} (Fig. 3-6c), and *E. coli* KO229 harboring pNTRm2-1 or pNTRm6-1 produced mainly CoQ_{10}, with small amounts of CoQ_9 and CoQ_8 (Fig. 3-6d, e). The *ispB* gene is essential for *E. coli* growth and is responsible for CoQ side chain length determination (Okada et al. 1997b), and these results clearly indicate that Dps1 proteins from *S. complicata* and *R. minuta* have deca-PDS activity and can act alone in *E. coli* to complement *ispB* (see also Fig. 3-7).

Attempts were made to exchange pKA3 with pNTB1-1, which encoded *B. albus* Dps1, but these were unsuccessful. In this case, Dps1 activity level may be insufficient to support growth.

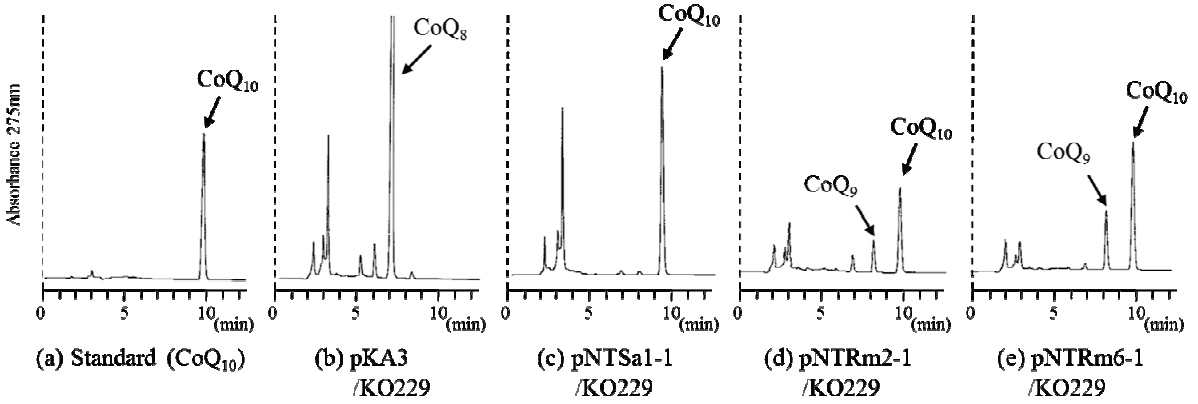

Fig. 3-6 Detection of CoQ in *E. coli* KO229 expressing fungal *dps1* genes

The *dps1* genes from *B. albus*, *S. complicata*, and *R. minuta* were expressed in *E. coli* KO229, an ispB-defective mutant. CoQ was extracted and analyzed by HPLC. Standard CoQ_{10} (a), and CoQ from KO229 harboring pKA3 (*ispB*) (b), KO229 harboring pNTSa1-1 (c), KO229 harboring pNTRm2-1 (d), and KO229 harboring pNTRm6-1 (e) are shown.

Enzymatic activity of fungal Dps1

To determine whether the recombinant proteins exhibited any specific enzymatic activity, *in vitro* assays were performed with IPP and FPP as allylic substrates. Enzymatic activity was measured by determination of the amount of [1-^{14}C]IPP incorporated into polyprenyl diphosphates. The product generated in the reaction was hydrolyzed by acid phosphatase and separated by reverse-phase TLC. As shown in Fig. 3-7, the major product of wild-type *E. coli* DH5α was octaprenol. *E. coli* KO229 harboring pNTSa1-1, pNTRm2-1, or pNTRm6-1 instead produced solanesol and decaprenol. Decaprenol was detected in all three recombinants, verifying that the fungal Dps1 proteins were active in *E. coli* and had deca-PDS activity.

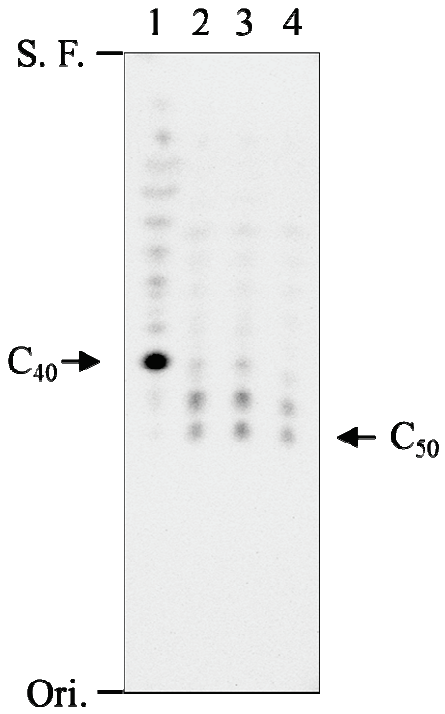

Fig. 3-7 Reversed-phase thin-layer chromatogram of products from recombinant *E. coli* KO229 strains
Cell extracts were obtained from *E. coli* KO229 expressing fungal dps1 genes and used for enzyme assays with [1-^{14}C]IPP and FPP as substrates. Reaction products were hydrolyzed by acid phosphatase, and the resulting alcohols were analyzed by reversed-phase TLC. *Arrowheads* indicate positions of C40 (octaprenol) and C50 (decaprenol). *Lane 1*, KO229 harboring pKA3 (*ispB*); *lane 2*, KO229 harboring pNTRm2-1; *lane 3*, KO229 harboring pNTRm6-1; and *lane 4*, KO229 harboring pNTSa1-1. *Ori.* origin, *S. F.* solvent front.

Complementation of a fission yeast dps1 and dlp1 disruptant with fungal dps1 genes

CoQ biosynthesis in S. pombe is performed by a heterotetrameric deca-PDS composed of Dps1 and Dlp1 (Saiki et al. 2003a). Disruption of either the *dps1* or the *dlp1* gene causes a severe growth delay when *S. pombe* is grown on minimal medium. The phenotype can be recovered by introducing a complementary gene such as *ddsA* from *G. suboxydans*, which encodes deca-PDS (Saiki et al. 2003a). To examine the ability of *dps1* genes from *B. albus*, *S. complicata*, and *R. minuta* to complement deca-PDS deficiency in fission yeast, Dps1 proteins

were expressed in CoQ-deficient mutant LA1 (*Δdps1*, *Δdlp1*). Four plasmids were constructed and introduced into LA1 (*Δdps1*, *Δdlp1*): pREP1-B1dps1, which expressed *B. albus dps1*; pREP1-Sa1dps1, which expressed *S. complicata dps1*; pREP1-Rm2dps1, which expressed *R. minuta dps1*; and pREP1-Rm6dps1, which expressed shortened *R. minuta dps1* (Fig. 3-4B). The growth delay phenotype of LA1 on minimal medium was rescued by all three *dps1* genes. Transformant growth was at levels close to that of LA1 harboring pREP1-dps1-dlp1, which expressed *S. pombe dps1* and *dlp1* (Fig. 3-8A). CoQ was extracted from transformants and analyzed by HPLC. LA1 harboring pREP1-B1dps1, pREP1-Sa1dps1, pREP1-Rm2dps1, or pREP1-Rm6dps1 all produced CoQ_{10} as the major product, although amounts of CoQ_{10} varied between transformants (Fig. 3-8B). The observation that the *dps1* genes from *B. albus*, *S. complicata*, and *R. minuta* could complement *S. pombe* LA1 (*Δdps1*, *Δdlp1*) and restore production of CoQ_{10} indicated that Dps1 from these species functioned as homomeric rather than as heteromeric enzymes.

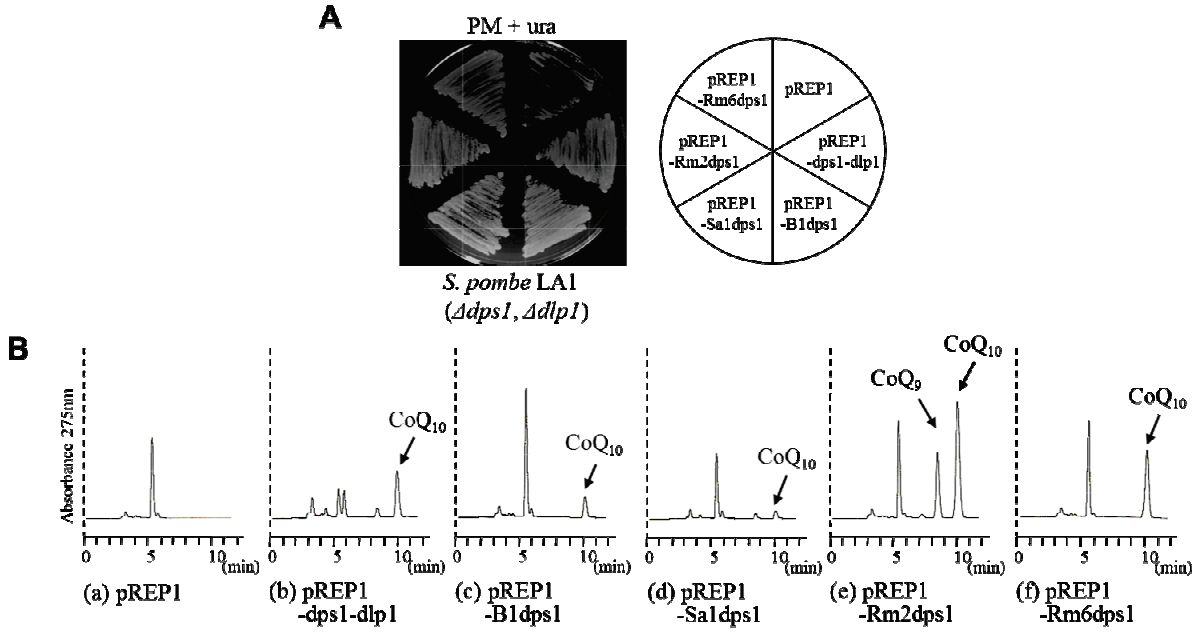

Fig. 3-8 Complementation of *S. pombe* LA1 (*Δdps1*, *Δdlp1*) with fungal *dps1* genes
A Growth of LA1 (*Δdps1*, *Δdlp1*) on minimal medium was observed. LA1 harboring the indicated plasmids was grown for 4 days at 30 ˚C on PM minimal medium containing uracil. **B** CoQ species in fission yeast LA1 expressing fungal *dps1* genes derived from *B. albus*, *S. complicata*, and *R. minuta* was analyzed by HPLC. CoQ was extracted from LA1 harboring plasmid pREP1 (a), pREP1-dps1-dlp1 (b), pREP1-B1dps1 (c), pREP1-Sa1dps1 (d), pREP1-Rm2dps1 (e), and pREP1-Rm6dps1 (f).

CoQ_{10} productivity of S. pombe expressing fungal dps1 genes

S. pombe naturally produces CoQ_{10}, and biosynthesis of CoQ_{10} in this species is a subject of increasing recent research interest. (Hayashi et al. 2014). I wished to determine whether CoQ_{10} production in *S. pombe* could be increased by expressing *dps1* genes from other fungi. Four plasmids were used to introduce the *dps1* genes into fission yeast: pREP1-B1dps1, pREP1-Sa1dps1, pREP1-Rm2dps1, and pREP1-Rm6dps1. Plasmids were introduced into wild-type *S. pombe* PR110 and CoQ production was quantified by using CoQ_6 as an internal standard. The production of CoQ was evaluated per culture volume and dry cell weight (DCW) (Fig. 3-9). No increase or decrease in CoQ_{10} production was seen statistically with any of the *dps1* plasmids.

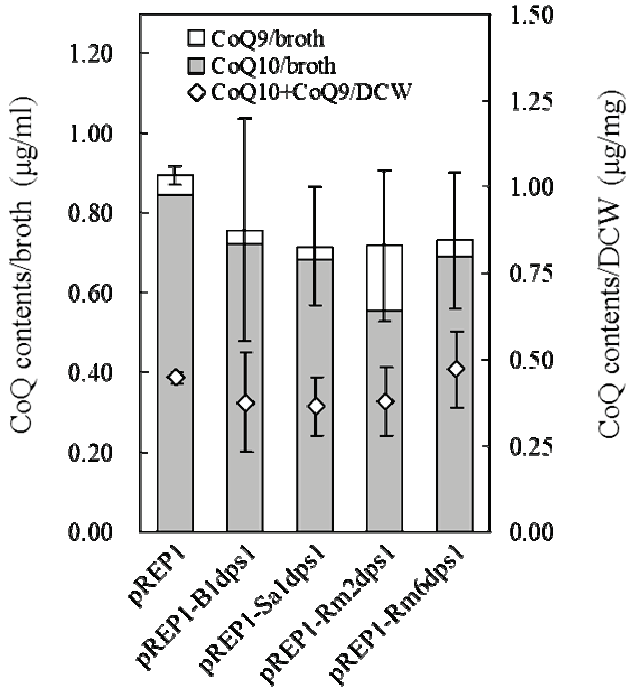

Fig. 3-9 Effect of fungal *dps1* gene expression on CoQ_{10} production in fission yeast.

CoQ productivity in strains expressing fungal *dps1* genes was compared to that in reference strain PR110 (pREP1). Strains were grown at 30 ˚C in PM minimal medium containing uracil. Cultures were initiated at 1 × 10^5 cells/ml and harvested after 48-h growth. Production of CoQ_{10} was measured by HPLC. *Gray bars* represent CoQ_{10} content per volume (%), *white bars* represent CoQ_9 content per volume (%), and *open diamonds* represent total CoQ (CoQ_{10} + CoQ_9) content per dry cell weight (DCW) (%). *Error bars* represent the standard deviation of three measurements of total CoQ.

Discussion

Deca-PDSs are responsible for the synthesis of the 10-unit isoprene side chain in CoQ_{10}. CoQ_{10} is a commercially popular food supplement, and identifying and isolating biosynthetic genes from CoQ_{10}-producing microorganisms are valuable for enhancing future CoQ_{10} production. In this study, the genes encoding deca-PDSs in three CoQ_{10} producing fungi, *B. albus*, *S. complicata*, and *R. minuta*, were cloned and characterized.

Long-chain trans-prenyl diphosphate synthases such as deca-PDS are classified into homodimer and heterotetramer types. *IspB* from *E. coli* (Kainou et al. 2001) and SPS1 from *Arabidopsis* (Jun et al. 2004) are homodimers. Heterotetramers include the deca-PDSs Dps1 and Dlp1 from *S. pombe* (Saiki et al. 2003a) and humans (Saiki et al. 2005). Predicted amino acid sequences for the fungal Dps1 proteins obtained in this study are highly similar to *E. coli* IspB, *S. pombe* Dps1, and human DPS1. The sequence similarities of *B. albus* Dps1, *S. complicata* Dps1, and *R. minuta* Dps1 with *S. pombe* Dps1 were 50, 51, and 46%, respectively. The three fungal Dps1 proteins contained seven conserved regions (domains I–VII) typically found in long-chain trans-prenyl diphosphate synthases, and exhibited common DDXXD motifs for FPP and IPP recognition. An unusually long stretch of amino acid residues was seen between domains I and II in *B. albus* Dps1. Divergence between the proteins was substantial at the N terminus. This region is absent in *E. coli* IspB and is thought to contain signal sequences responsible for subcellular localization (likely for mitochondria). CoQ is synthesized in mitochondria in *S. cerevisiae* (Tran and Clarke 2007) and *S. pombe* (Hayashi et al. 2014), and it is reasonable to suggest the same synthesis location in other fungi. As *B. albus* and *S. complicata* genome sequences are now available (Nishida et al. 2011), I searched for the sequence of *dps1* and found there is an almost identical gene (only three nucleotide differences) in *B. albus* and a completely identical gene in *S. complicata*, but I did not find *dlp1* like genes in both species.

The fungal dps1 genes were expressed in wild-type *E. coli*, and all the resultant transformants produced CoQ_{10} in addition to CoQ_8. These findings indicated that the Dps1 enzymes possessed deca-PDS activity and functioned as homomeric proteins. This was confirmed by expressing the *S. complicata* and R. minuta *dps1* genes in *E. coli* KO229, which lacks the chromosomal *ispB* gene. *E. coli* KO229 can not survive without a plasmid carrying *ispB* or an episomal counterpart gene. The *ispB*-carrying plasmid was successfully replaced with plasmids carrying *dps1* from *S. complicata* or *R. minuta*, but not from *B. albus*. This suggested that the enzyme activity produced by *B. albus* Dps1 was insufficient to replace the

activity of endogenous *ispB*. Enzymatic activities producing decaprenyl diphosphate were detected in KO229 expressing *S. complicata* and *R. minuta dps1*. Solanesyl diphosphate was produced in an *in vitro* reaction and CoQ_9 was produced in KO229 expressing *R. minuta*. *R. minuta* produces CoQ_{10}, and it is likely that a heterologous expression system alters the enzymatic reaction. Expression of *B. albus*, *S. complicata*, or *R. minuta dps1* in *S. pombe* CoQ-deficient mutant LA1 (*Δdps1*, *Δdlp1*), fully abrogated a growth delay phenotype on minimal medium, and CoQ_{10} was the major product in these cells. The data from *E. coli* and *S. pombe* consistently show that the three fungal *dps1* genes cloned in this study produce proteins that are independently active in *E. coli* and *S. pombe* and have deca-PDS activity. In some cases, heterologous expression of Dps1 produced CoQ_9 in addition to the main CoQ_{10} product. I suggest that the enzyme activity of Dps1 was insufficient in the heterologous system, leading to release of a shorter length of isoprenyl diphosphate and thereby producing CoQ with a shorter tail length.

Recent studies explored CoQ_{10} biosynthesis in *S. pombe* (Hayashi et al. 2014), and CoQ_{10} productivity was increased (Moriyama et al. 2015). However, expression of endogenous *dps1* did not lead to an increase CoQ_{10} production, probably as a result of cell growth inhibition from localization of large amounts of autologous protein at the mitochondria (Moriyama et al. 2015). Here, I expressed exogeneous *dps1* genes from *B. albus*, *S. complicata*, and *R. minuta* in *S. pombe*; however, while no growth inhibition was seen, no increase in CoQ yield was observed. It is possible that exogeneous Dps1 interferes with endogenous *S. pombe* Dps1 or Dlp1. Heterologous protein interactions were observed previously between *S. cerevisiae* Coq1 and *S. pombe* Dps1 (Zhang et al. 2008a) and between *E. coli* IspB and *S. pombe* Dps1 (Cui et al. 2010) and, although these were positive interactions, it can be assumed that negative interactions are also possible. If exogeneous Dps1 interfered with endogenous *S. pombe* Dps1 or Dlp1, production of CoQ_{10} would not increase even though exogenous Dps1 is active by itself or may decrease.

In summary, *dps1* genes from the fungi *B. albus*, *S. complicata*, and *R. minuta* were successfully cloned. The genes were expressed in *E. coli* and *S. pombe*, and the Dps1 proteins possessed deca-PDS activity and functioned as homomeric enzymes. Those cloned *dps1* genes can be used to enhance efficient production of CoQ_{10} in a range of species.

Chapter 4

Conclusions

Coenzyme Q (CoQ), also refers to as ubiquinone, is a well known component of the electron transport chain that participates in aerobic cellular respiration within mitochondria and is essential for ATP-dependent energy production. CoQ consists of a hydrophobic isoprenoid side chain and a quinone ring. CoQ delivers electrons through the conversion of quinol (reduced form) and quinone (oxidized form). CoQ acts as a fat-soluble antioxidant by this oxidation-reduction reaction, which contributes to the removal of lipid peroxidation. It is known that the amount of CoQ_{10} synthesis of human decreases after peaking at 20 years old, so that the external supplementation of CoQ_{10} is considered to be important for keeping an active life. So currently, the intake of CoQ_{10} by supplements has become popular and therefore the demand for CoQ_{10} has been increasing.

Living organisms possess different species of CoQ depending on the length of the isoprenoid side chain. For example, humans and *S. pombe* produce CoQ harboring ten-unit isoprene (CoQ_{10}), *E. coli* produces CoQ_8, and *S. cerevisiae* produces CoQ_6. Since *S. pombe* naturally produces CoQ_{10}, it is easier to manipulate gene expression to produce CoQ_{10} than other popular organisms such as *S. cerevisiae* and *E. coli*, which only produce CoQ_6 and CoQ_8, respectively. In this research, I aim to improve the productivity of CoQ_{10} in *S. pombe* which naturally produces CoQ_{10}, and look for decaprenyl diphosphate synthase genes useful for production of CoQ_{10} in heterologous hosts.

Firstly, in order to improve the CoQ_{10} productivity in the fission yeast, CoQ biosynthetic genes (*dps1-dlp1*, *ppt1*, *coq3-coq9*) was cloned and individually overexpressed in *S. pombe*, however, the CoQ productivity hardly changed. As neither one of the CoQ biosynthetic genes may not be rate-limiting, I therefore simultaneously enhanced the expression of multiple CoQ biosynthetic genes. However, the CoQ productivity was not improved in spite of 10 kinds of CoQ biosynthetic genes were simultaneously enhanced. I then attempted to improve CoQ_{10} productivity by increasing the supply of the CoQ_{10} precursors PHB and DPP. Nine different biosynthetic genes involved in the shikimate and mevalonate pathways were overexpressed in *S. pombe*. The overexpression of chorismate lyase from *E. coli* (*Eco_ubiC*) or truncated HMG-CoA reductase from *S. cerevisiae* (*Sce_thmgr1*) gene resulted in a CoQ_{10} productivity increase of approximately 30% and the overexpression of the feedback-inhibition-resistant DAHP synthase from *E. coli* (*Eco_aroF*^{FBR}) gene increased productivity by approximately 15%. Furthermore, co-expression of these upstream genes resulted in two-fold CoQ_{10} production.

Secondly, three deca-PDS genes from three CoQ_{10} producing fungi, *B. albus*, *S. complicata*,

and *R. minuta* were cloned. The predicted Dps1 proteins contained seven conserved regions (domains I-VII) found in typical polyprenyl diphosphate synthases and were 528, 440, and 537 amino acids in length in *B. albus*, *S. complicata*, and *R. minuta*, respectively. The sequence similarities of *B. albus* Dps1, *S. complicata* Dps1, and *R. minuta* Dps1 with *S. pombe* Dps1 were 50, 51, and 46%. I and my collaborators confirmed that these three fungal Dps1 enzymes possessed deca-PDS activity and functioned as homomeric proteins, and these *dps1* genes complemented *E. coli* KO229 (*ΔispB*) and *S. pombe* LA1 (*Δdps1*, *Δdlp1*) strains, both of which lack polyprenyl diphosphate synthase. Furthermore, I tested the CoQ_{10} production in *S. pombe* was increased by expressing exogeneous *dps1* genes from *B. albus*, *S. complicata*, and *R. minuta*; however, no increase in CoQ_{10} yield was observed. It is possible that exogenous Dps1 interferes with endogenous *S. pombe* Dps1 or Dlp1.

In summary, I aimed to improve the productivity of CoQ_{10} in *S. pombe* by expression of a panel of biosynthetic genes. CoQ productivity was not improved by overexpression of CoQ biosynthetic genes, but it was improved by increasing the supply of the CoQ_{10} precursors PHB and DPP by enhancement of upstream responsible genes. By cloning novel decaprenyl diphosphate synthases from three fungi, I proved that they are functional in *S. pombe.* These findings in this thesis will help the improvement CoQ_{10} productivity in *S. pombe* or other species.

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…….Chapter 2

(2) Moriyama D, Kaino T, Yajima K, Yanai R, Ikenaka Y, Hasegawa J, Washida M, Nanba H, Kawamukai M (2016) Cloning and characterization of decaprenyl diphosphate synthase from three different fungi. Appl Microbiol Biotechnol 101:1559-1571

…….Chapter 3

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Summary

Coenzyme Q (CoQ), also called ubiquinone, is a well known component of the electron transport chain that participates in aerobic cellular respiration within mitochondria and is essential for ATP-dependent energy production. CoQ consists of a hydrophobic isoprenoid side chain and a quinone ring, CoQ delivers electrons through the conversion of quinol (reduced form) and quinone (oxidized form). CoQ acts as a fat-soluble antioxidant by this oxidation-reduction reaction, which contributes to the removal of lipid peroxidation, and CoQ plays the role of the electron donor during disulfide bond formation in *Escherichia coli*, and its reduction is coupled to sulfide oxidation in *Schizosaccharomyces pombe* and other organisms. Moreover, CoQ is required for *de novo* synthesis of UMP (uridine monophosphate) in many eukaryotes. It is known that the amount of CoQ_{10} synthesis of human decreases after peaking at 20 years old, so that the external supplementation of CoQ_{10} is considered to be important for keeping an active life. So currently, the intake of CoQ_{10} by supplements has become popular and therefore the demand for CoQ_{10} has been increasing.

Living organisms possess different species of CoQ depending on the length of the isoprenoid side chain. For example, humans and *S. pombe* produce CoQ harboring ten-unit isoprene (CoQ_{10}), *E. coli* produces CoQ_8, and *Saccharomyces cerevisiae* produces CoQ_6. Since *S. pombe* naturally produces CoQ_{10}, it is easier to manipulate gene expression to produce CoQ_{10} than other popular organisms such as *S. cerevisiae* and *E. coli*, which only produce CoQ_6 and CoQ_8, respectively. In this research, I aim to improve the productivity of CoQ_{10} in *S. pombe* which naturally produces CoQ_{10}, and look for decaprenyl diphosphate synthase genes useful for production of CoQ_{10} in heterologous hosts.

In chapter 1, the author described the general introduction of the thesis.

In chapter 2, I aimed to improve the productivity of CoQ_{10} in *S. pombe* by expression of a panel of biosynthetic genes. In order to improve the CoQ_{10} productivity, CoQ biosynthetic genes (*dps1-dlp1*, *ppt1*, *coq3-coq9*) were cloned and individually overexpressed in *S. pombe*, however, the CoQ productivity hardly changed. As neither one of the CoQ biosynthetic genes may not be rate-limiting, I therefore simultaneously enhanced the expression of multiple CoQ biosynthetic genes. However, the CoQ productivity was not improved in spite of 10 kinds of CoQ biosynthetic genes were simultaneously enhanced. I then attempted to improve CoQ_{10} productivity by increasing the supply of the CoQ_{10} precursors PHB (*p*-hydroxybenzoate) and

DPP (decaprenyl diphosphate). Nine different biosynthetic genes involved in the shikimate and mevalonate pathways were overexpressed in *S. pombe*. These include the genes which lost its regulation that is known to regulate the mevalonate and shikimate pathway, and the genes allow the efficient flow bypassing metabolic pathway. The overexpression of chorismate lyase from *E. coli* (*Eco_ubiC*) or truncated HMG-CoA reductase from *S. cerevisiae* (*Sce_thmgr1*) gene resulted in a CoQ_{10} productivity increase of approximately 30% and the overexpression of the feedback-inhibition-resistant DAHP (3-deoxy-D-arabinoheptulosonate 7-phosphate) synthase from *E. coli* (*Eco_aroF*^{FBR}) gene increased productivity by approximately 15%. Furthermore, the result of co-expression of these upstream genes, two-fold increase of a CoQ_{10} productivity was observed in the strains expressing *Sce_thmgr1* and *Eco_aroF*^{FBR}, *Sce_thmgr1* and *Eco_ubiC*. No further gain in productivity was observed when all three genes (*Sce_thmgr1*, *Eco_aroF*^{FBR}, and *Eco_ubiC*) were co-expressed. These results indicate that, in *S. pombe*, the overproduction of precursors in the CoQ biosynthetic pathway is an effective strategy for improving CoQ productivity.

In chapter 3, three decaprenyl diphosphate synthase genes from three CoQ_{10} producing fungi, *Bulleromyces albus*, *Saitoella complicata*, and *Rhodotorula minuta* were cloned. The predicted Dps1 proteins contained seven conserved regions (domains I-VII) typically found in long-chain trans-prenyl diphosphate synthases, and exhibited common DDXXD motifs for FPP (farnesyl diphosphate) and IPP (isopentenyl diphosphate) recognition. These fungal Dps1 were 528, 440, and 537 amino acids in length in *B. albus*, *S. complicata*, and *R. minuta*, respectively. The sequence similarities of *B. albus* Dps1, *S. complicata* Dps1, and *R. minuta* Dps1 with *S. pombe* Dps1 were 50, 51, and 46%. I and my collaborators characterized these three fungal Dps1 enzymes in *E. coli* and *S. pombe*. *E. coli* expressing the fungal *dps1* genes produced CoQ_{10} in addition to endogenous CoQ_8. Two of the three fungal *dps1* genes (from *S. complicata* and *R. minuta*) were able to replace the function of *ispB* in an *E. coli* mutant strain. *In vitro* enzymatic activities were also detected in recombinant strains. The three *dps1* genes were able to complement a *S. pombe dps1*, *dlp1* double mutant. Recombinant *S. pombe* produced mainly CoQ_{10}, indicating that the introduced genes were independently functional and did not require *dlp1*. Furthermore, I tested whether the CoQ_{10} production in *S. pombe* was increased by expressing exogeneous *dps1* genes from *B. albus*, *S. complicata*, and *R. minuta*; however, no increase in CoQ_{10} yield was observed. It is possible that exogeneous Dps1 interferes with endogenous *S. pombe* Dps1 or Dlp1. These fungal Dps1 proteins possessed deca-PDS activity and functioned as homomeric enzymes, and the cloned *dps1* genes can be used to enhance

efficient production of CoQ_{10} in a range of species.

From the results, I conclude two things: (1) CoQ productivity was not improved by overexpression of CoQ biosynthetic genes, but it was improved by the supply of the CoQ_{10} precursors PHB and DPP by enhancement of upstream responsible genes. (2) By cloning novel decaprenly diphosphate synthases from three fungi, I proved that they are functional in *S. pombe*.

要約

コエンザイムQ（CoQ）はユビキノンとも称され、ミトコンドリアにおける好気性呼吸に関与する電子伝達系の構成成分であり、ATP依存型のエネルギー生産において重要な役割を果たしている。CoQはイソプレノイド側鎖とキノン環からなり、キノン（酸化型）とキノール（還元型）の変換を行うことで電子の受け渡しを行う。この酸化還元反応によりCoQは脂溶性抗酸化物質として働き、過酸化脂質の除去に寄与している。またCoQは、大腸菌（*Escherichia coli*）において、タンパク質のジスルフィド結合の際の電子供与体としての役割を果たしたり、分裂酵母（*Schizosaccharomyces pombe*）やその他の生物で広く硫化物の酸化に関与することが知られている。さらにCoQは、真核生物のUMP（uridine monophosphate）の*de novo*合成にも必要であることが知られている。しかしながら、ヒトのCoQ_{10}合成量は20歳をピークに減少することが知られており、外部からCoQ_{10}を補給することは重要であると考えられている。そのため、現在ではサプリメントでのCoQ_{10}の摂取が一般的となり、CoQ_{10}の需要は増加の一途をたどっている。

CoQは生物種によってそのイソプレノイド側鎖の鎖長が異なり、*S. pombe*やヒトではCoQ_{10}, *E. coli*ではCoQ_8, 出芽酵母（*Saccharomyces cerevisiae*）ではCoQ_6を合成する。*S. pombe*は元々CoQ_{10}を生合成するため、CoQ_6やCoQ_8を生合成する他の一般的な微生物である*S. cerevisiae*や*E. coli*と比較して、遺伝子操作によるCoQ_{10}生産が容易である。そこで本研究では、元々CoQ_{10}を生産する*S. pombe*の遺伝子組換えによるCoQ_{10}生産性向上の検討と、異種の宿主でのCoQ_{10}生産に有用な新規なデカプレニル二リン酸合成酵素の探索を行った。

第１章では本論分のバックグラウンドについて述べた。

第２章において、*S. pombe*でCoQ生合成遺伝子群を発現させCoQ_{10}生産性の向上を試みた。*S. pombe*からCoQ生合成遺伝子（*dps1-dlp1*, *ppt1*, *coq3-coq9*）をクローニングし、個々に高発現させたがCoQ生産性にほとんど変化は認められなかった。そこで、増強したCoQ生合成遺伝子とは異なる遺伝子が律速となっている可能性を考え、複数のCoQ生合成遺伝子の同時増強を試みた。しかしながら、10種のCoQ生合成遺伝子を同時増強したにもかかわらず、CoQ_{10}の生産性向上は認められなかった。次に、CoQ_{10}の前駆体であるPHB（*p*-hydroxybenzoate）及び、DPP（decaprenyl diphosphate）の供給量を増やすことでCoQ_{10}の生産性が向上しないか検討を行った。シキミ酸経路やメバロン酸経路を調節していることが知られている遺伝子のうち、それらの制御を解除し

た遺伝子や、生合成経路をバイパスし効率的な代謝の流れを可能にする遺伝子の計９種についてクローニングを行い、各種遺伝子の単独増強株を取得してCoQ_{10}の生産性を評価した。その結果、*Eco_ubiC*（*E. coli*由来のchorismate lyase）及び、*Sce_thmgr1*（*S. cerevisiae*由来の切断型HMG-CoA reductase 1）遺伝子増強株で約30%、*Eco_aroF*^{FBR}（*E. coli*由来のフィードバック阻害耐性DAHP（3-deoxy-D-arabinoheptulosonate 7-phosphate）synthase）遺伝子増強株で約15%のCoQ_{10}の生産性向上効果が認められた。そこでさらに、これらの遺伝子の同時増強について検討を行った結果、*Sce_thmgr1*と*Eco_aroF*^{FBR}、*Sce_thmgr1*と*Eco_ubiC*の２遺伝子増強株、及び、*Sce_thmgr1*と*Eco_aroF*^{FBR}と*Eco_ubiC*の３遺伝子増強株で２倍のCoQ_{10}生産性向上効果が認められた。これらの結果より、*S. pombe*において、CoQ_{10}前駆体の供給量増加がCoQ_{10}の生産性向上に効果的であることが示唆された。

第３章において、CoQ_{10}を生合成する酵母*Bulleromyces albus*、*Saitoella complicata*、*Rhodotorula minuta*から３種のデカプレニル二リン酸合成酵素遺伝子をクローニングした。これらの酵素Dps1には長鎖trans-プレニル二リン酸合成酵素に典型的な７つの保存された領域（ドメインI-VII）が認められ、基質であるFPPとIPPの認識にかかわる共通配列“DDXXD”も認められた。また、*S. complicata*、*B. albus*、*R. minuta*のDps1は、それぞれ440、528、537アミノ酸をコードしており、CoQ_{10}を生合成する*S. pombe*のDps1と比較して、それぞれ51%、50%、46%の相同性があった。そこで著者らは、これらの酵母由来Dps1を*E. coli*及び*S. pombe*で発現させ、その性質を調べた。その結果、これらのDps1を発現させた*E. coli*ではもともとのCoQ_8に加えてCoQ_{10}を生合成することが確認でき、３種の酵母由来*dps1*遺伝子うち２つ（*S. complicata*と*R. minuta*由来）について、*E. coli*の側鎖合成酵素遺伝子である*ispB*を相補可能であることが確認できた。また、*S. pombe*においても３種の酵母由来*dps1*遺伝子が*S. pombe*の側鎖合成酵素遺伝子*dps1*と*dlp1*を相補可能であることが確認できた。さらに、これらの３種類の*dps1*遺伝子を*S. pombe*で増強しCoQ_{10}の生産性向上が可能か検討したが、生産性の向上は認められなかった。外来のDps1が*S. pombe*のDps1又はDlp1と相互作用し悪影響を及ぼしている可能性を考えている。これらの結果より、酵母由来の３種のDps1がデカプレニル二リン酸合成酵素活性を有し、ホモマーとして機能していることが確認できた。今回クローニングした酵母由来*dps1*遺伝子は*S. pombe*やその他の生物種でのCoQ_{10}の効率的生産に有用と考えられる。

これらの結果より、著者は以下のことを明らかにした。（１）CoQ 生合成遺伝子の増強では CoQ_{10} 生産性の向上は認められないが、生合成経路上流の遺伝子を増強して CoQ_{10} の前駆体である PHB 及び DPP の供給量を増加させることが CoQ_{10} の生産性向上に

効果的であることを確認した。（２）３種の酵母より新規のデカプレニル二リン酸合成酵素をクローニングし、*S. pombe* 内で機能することを証明した。